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CHEMICAL COUPLING OF GLUCOAMYLASE PRODUCED BY *Arthrobotrys conoides* ONTO COTTON CLOTH AND *Ocimum basilicum* SEEDS AND CHARACTERIZATION OF THE IMMOBILIZED ENZYME

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ABSTRACT

Glucoamylase produced by *Arthrobotrys conoides* was immobilized onto cotton cloth pieces (CC) and seeds of *Ocimum basilicum* (OB) employing five different approaches. Mechanical stability of CC and large surface area provided by the microfibrillar structure in the mucilage of OB seeds offers the advantage for their use as immobilization matrices. Periodate treated enzyme coupled to polyethylenimine treated support gave the best results with immobilization percentage of 67.5 and 53 for CC and OB seeds respectively. Immobilized enzymes exhibited broader pH and temperature activity profiles as compared to those of native and PI oxidized forms. Immobilization conferred stability to the enzyme in acidic region and also improved its thermo-stability. Km values for starch were found to be 0.08 and 0.105 $mg.ml^{-1}$ for native and PI treated forms and, 2.3 and 2.6 $mg.ml^{-1}$ for enzymes bound to CC and OB respectively. Although the enzyme preparations were optimally active at 50°C, recycling studies indicated optimum temperature of 40°C for saccharification of starch. CC and OB bound preparations could be recycled 13 and 11 times respectively every 2h at 40°C, with retention of 50% activity. Immobilized preparations were able to convert starch to an extent of 61-64%. Conversion percentage improved to 73% when CC preparation was incubated with starch at increased speed of agitation indicating diffusional limitations as one of the factors influencing the apparent decrease in the affinity of the immobilized enzyme for its substrate. Cloth bound preparation was found to be superior in its performance in comparison to enzyme coupled to OB.

Keywords: Glucoamylase, Immobilization, Cotton, *Ocimum basilicum*, periodate treatment, polyethylenimine treatment

INTRODUCTION

Glucoamylases ((EC 3.2.1.3.) are exo acting α-1,4-glucan glucohydrolases which act on glucan polysaccharides yielding glucose as the major product. The enzyme is used by industries involved in saccharification of starch for glucose and alcohol production. *Arthrobotrys* species are a group of nematophagous fungi, which kill and consume microscopic animals. These fungi are known to produce pectinases, amylases, cellulases and various other hydrolytic enzymes. We have reported production of glucoamylase by *Arthrobotrys* species (**Jaffar *et al.*, 1993; Shetty, 2016**). Immobilization of glucoamylase has been widely studied as a means of reducing enzyme cost in the manufacture of glucose and high fructose corn syrups. Various supports and techniques have been assessed for immobilization. Activated alginate beads, ion exchangers, chitosan, earth materials and equivalents, magnetic micro-particles etc. have served as matrices for immobilization of amylolytic enzymes (**Pieters *et al.*, 1992; Goncalves *et al.*, 1997; Iyer *et al.*, 2003; Shkutina *et al.*, 2005; Eldin, *et al.*, 2011; Wang *et al.*, 2013**). Choice of a suitable matrix and technique of binding are of critical importance in immobilization. Attrition and/ flotation are often the problems associated in the long run in agitated reactors using shear sensitive support materials (**Regan *et al.*, 1974**). Particulate supports have the limitations of high pressure drop and hindered flow characteristics in packed bed reactors on continuous use (**Svec & Gemeiner, 1995; Suen, 2015**). Methods of immobilization such as adsorption and entrapment, though simple and efficient, do not create strong bonds between the enzyme and the support and often, enzyme leaks into the solution. Diffusional restrictions encountered further limit the benefit of entrapment systems which act on polymeric substrates. Covalent binding is a promising technique which takes care of such issues associated with adsorption and entrapment. One of the key issues associated with covalent linkage is the need for activation the support and often modification of the enzyme as well, which may result in loss of activity to a significant extent. Glucoamylases are glycoproteins by nature (**Shenoy *et al.*, 1985**) Binding of the enzyme through its glycosyl residues can be a method of choice for immobilization.

The present investigation deals with immobilization of glucoamylase produced by *A conoides* onto cotton cloth pieces and the seeds of *Ocimum basilicum* (locally known as Sabja). Various techniques were evaluated for immobilization. The enzyme bound preparations were characterized and assessed for their recycling and saccharification efficiency.

MATERIALS AND METHODS

Microorganism

The organism *A conoides* (ATCC 44454) was maintained on corn meal agar slants as recommended by ATCC catalogue, 1983.

Enzyme production

Enzyme was produced in a medium containing ($g.l^{-1}$), pH 7.2: Corn starch, 10.0; peptone, 2.0; $NaNO_3$, 3.7; KH_2PO_4, 3.4; KCL and $MgSO_4.7H_2O$, 0.5g each; $FeSO_4.7H_2O$, 10 mg; $ZnSO_4.7H_2O$, 0.5 mg, thiamine HCl, 0.1 mg and biotin, 5µg. The medium (25 ml) was dispensed into 250 ml Erlenmeyer flasks and inoculated with around 10^6 spores. Incubation was carried out at 25±1°C for 12 days under stationary conditions. The broth was filtered and the culture filtrate was used for further studies.

Assay methods

Amylolytic activity of the free enzyme was measured in 2 ml of the reaction mixture containing appropriately diluted enzyme and 0.25% soluble starch in 25 mM acetate buffer, pH 5.6. It was then incubated at 40°C for 20min and the resultant reducing sugars were measured by DNSA. Glucose produced was estimated using glucose oxidase-peroxidase reagent (GOP method). The assays were carried out by DNSA method unless mentioned otherwise.

Immobilized activity was measured by addition of 5 ml of soluble starch (1.5% in 25mM acetate buffer, pH 5.6), to the immobilized support. After an incubation period of 1h at 40°C in a shaker water bath at 40 rpm, reducing sugars were estimated in the supernatant by DNSA method.

Unit of activity is defined as the µmole of reducing equivalent (glucose) released per minute under the assay conditions.
Protein was estimated by Lowry's method.

Detection of reaction products by paper chromatography:

One unit of the enzyme was incubated in 2ml of 0.25% starch at 50°C. The aliquots containing 20µg of reducing sugars (as determined by DNSA) was loaded onto Whatman No.1 filter paper. Descending paper chromatography was carried out in n-butanol:pyridine: water (6:4:3) solvent system. Sugars were detected by dipping in 0.1% silver nitrate in acetone, followed by development of color with 1.4% NaOH and destaining in 5% $Na_2S_2O_3$ solution **(Touchstone and Dobbins, 1978).**

Concentration

The culture filtrate was dialyzed against PEG 20000 and further concentration was carried out by salting out at 80% saturation with ammonium sulfate at 4°C. This preparation was used for immobilization.

Treatment of the enzyme

Periodate (PI) treatment: To 95 ml of the sample containing 100units of the enzyme (specific activity of 3.92) in 0.05M acetate buffer, pH 5.6, 5 ml of 100mM sodium meta periodate was added and kept in dark for 3h. Ethylene glycol, 1 ml was then added and kept for 30min. The solution was dialyzed overnight in refrigerator.
Polyethylenimine (PEI) treatment: To 95ml of the sample containing 100units of the enzyme, 5ml of 4% PEI was added and the pH was adjusted to pH 7. After 3h, the reaction mixture was dialyzed.

Treatment of the supports

White Cotton cloth was washed, rinsed in D/W and air-dried. Cloth pieces were cut (4x4cm, 105-115mg) and used for immobilization. OB seeds, 0.2g each were soaked in D/W for 2h and water was filtered off using a strainer. The strainer was placed on a pad of filter paper to absorb excess water.
The supports were subjected to following treatments.
PEI treatment: To one cloth piece/o.2g of swollen OB, 5ml of 0.2% PEI, pH 7.0 (adjusted with HCl) as added. After incubating for 2h at ambient temperature (30±1°C), the supernatant was discarded and the treated support was rinsed with D/W.
PI treatment: The support was immersed in 5 ml of 100mM sodium meta periodate for 3h in dark and rinsed with D/W.

Immobilization of the enzyme

Different methods employed for investigation were as follows, Method I- The enzyme was added to PEI treated support; Method II- PI treated enzyme was added to PEI treated support; Method III- PI treated enzyme was added to PEI treated support. After 3h, the support was rinsed with D/W and treated with 5 ml of 0.5% glutaradehyde for 1h; Method IV- Untreated enzyme was applied onto PI treated support; Method V- PEI treated enzyme was applied onto PI treated support.
Enzyme 2U was added per piece of cloth/0.2 g of OB seeds. Contact time for immobilization in all the methods was 3h at 30±1°C. The supernatant was assayed for unbound activity. Enzyme bound support was assayed for immobilized activity. The supports were stored under moist conditions (in 0.2 ml D/W) in refrigerator when not in use.
Desorption studies: The enzyme bound support was incubated in 5 ml of 0.2M NaCl for 10min at 40rpm. Residual activity in the support was assayed.
Optimization of enzyme dosage: Different units of the enzyme ranging from 1.35 to 5.4 were subjected to Method II of immobilization.

Characterization of the enzymes

Effect of temperature and pH on the activity of the free enzymes/ immobilized preparation was studied.
Enzyme was incubated at 30°C for 2h each in 5 ml buffer at various pHs ranging from pH 4 to 12.5 (Acetate, phosphate and glycine-NaOH buffers) for pH stability studies. Residual activity was then assayed at pH 5.6. Thermal stability was tested by incubating the enzymes at temperatures ranging from 35 - 55°C for 60 min followed by assay.
The enzyme was incubated with varying concentration of starch and the Km was determined from M-M and L-B plots.

Saccharifiaction studies

Recycling efficiency of the immobilized preparation: Immobilized preparation was incubated in 5 ml of the 1.5% starch solution for 2h at 35-50°C for 2h. The reducing sugars in the supernatant was estimated. The enzyme bound support was washed and resuspended in fresh batches of substrate under similar conditions.

Continuous use of immobilized preparation for starch saccharification: The immobilized preparations (10 cloth pieces/2g of OB seeds) were added to 30 ml of 8% starch solution contained in a 250 ml beaker and placed in the water bath maintained at 40°C at 40/100rpm. Aliquots of 50-100µl were taken periodically and analyzed for reducing sugar content. Percent conversion was calculated based on the consideration that 0.9g of starch produces 1g of glucose on saccharification.
Storage stability: Immobilized preparations were stored at 10°C in moist state for various time periods from 0-40days and then assayed.

RESULTS AND DISCUSSION

Concentration of the enzyme

Amylolytic activity elaborated by the fungus *A conoides* was assayed by DNSA and GOP method for estimation of total reducing sugars and glucose respectively. More than 95% of the reducing sugars comprised of glucose proving that the enzyme is glucoamylase. Paper chromatography results also proved that the major product of enzyme action was glucose (figure 1).

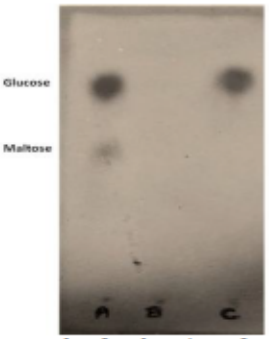

Figure 1 Paper chromatography for detection of reaction products. Lane A: Standards (glucose and maltose-20µg each); Lane B: 0 min; Lane C: 15min incubation

The yield of the enzyme was around 1.2U/ ml. The enzyme was concentrated by dialysing against PEG 20000, followed by salting out at 80% ammonium sulfate saturation. Concentrated enzyme was purified 3.26 folds with a specific activity of 3.92 and recovery of around 85%.

Immobilization of the enzyme

Five different approaches were employed for immobilization of the enzyme onto both the supports. **Melo *et al* (1986)** have suggested OB as a suitable pellicular support for immobilization of cells and enzymes. **Melo and D'Souza (1992)** have reported immobilization of invertase onto OB seeds. Sucrose, the substrate of invertase, is a small molecule with a molecular mass of 342 daltons. However, the substrate for amylase are starches which are large molecules. The average degree of polymerization of starch varies with origin and type, ranging from about 250-4000 anhydrous glucose units approximating to molecular masses of 40-650 kDa (Rutenberg, 1980). The performance of an immobilized enzyme acting on its high molecular weight substrate is affected to a great extent on the mode of attachment and rigidity conferred to the enzyme consequent to its binding. Immobilization of such enzymes to surface of the matrices may be advantageous to minimize diffusional limitations. Immobilization of glucoamylases, pectinases and cellulases onto activated surfaces of gels have been reported **(Tomar & Prabhu, 1985; Li *et al.*, 2008; Zhang et al., 2016).** Variety of supports with amino pendant groups have been used as enzyme carriers **(Yamazaki *et al.*, 1984; D'Souza & Godbole, 2002; Alahakoon *et al.*, 2012)**. Various supports have been converted into anion exchangers by treatment with PEI **(Bahulekar *et al.*, 1991; Wasserman *et al.*, 1982).** Proteins can bind to the PEI treated support via their negatively charged functional groups. Method I involved coupling of the untreated enzyme to PEI treated support. Glucoamylases are reportedly glycoproteins in nature **(Shenoy *et al.*, 1985)**. Glycoenzymes offer an opportunity to perform immobilization through their carbohydrate chains. Periodate (PI) ion is known to oxidize hydroxyl groups of adjacent carbon atoms in the glycosyl residues of the enzyme resulting in fission of the intervening C-C bond with formation of aldehyde groups **(Kiernan, 1990; Wong and Wong, 1992)**. The enzyme molecules can then be linked via the newly formed aldehyde groups through schiff's base to the PEI treated matrix. In Method II, PI treated enzyme was immobilized onto PEI coated support. An attempt was made in method III to further strengthen the binding using the bifunctional crosslinking reagent glutaraldehyde. The capsular mucilaginous layer of the OB is comprised of polysaccharides. As both the supports consist of carbohydrates, PI oxidation was employed as a strategy for activation of the supports in method IV. An attempt was made to couple the enzyme to PI treated support, wherein the aldehyde groups generated in the support were coupled to the amino groups of the enzyme. In method V, the amino pendant groups introduced into the enzyme by PEI treatment were coupled to PI treated carrier. The results are summarized in figure 2.

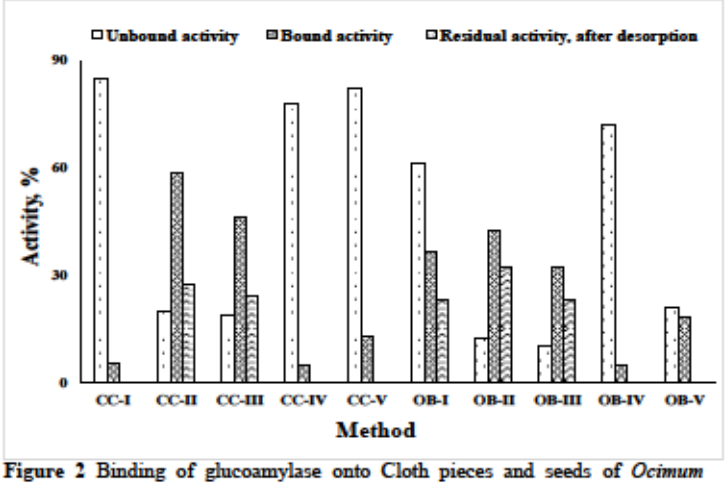

Figure 2 Binding of glucoamylase onto Cloth pieces and seeds of *Ocimum basilicum* by various techniques

Of the various techniques employed, methods II and III proved efficient for both supports. It was interesting to note that in methods III and V, the unbound fraction of OB exhibited only around 10.4 and 21% of activity respectively, implying that around 80-90% of activity was bound to the swollen seeds. However, only around 32.6% and 18.1% of activity was expressed as immobilized units. The pellicular nature of the support may have resulted in unproductive cross-linking between the enzyme and the support. In both the supports, maximum amount of immobilized units were obtained by methods II and III. In case of OB, method I also gave good yield of immobilized activity. These immobilized systems were subjected to desorption using 0.2M NaCl. As shown in fig.2, enzyme immobilized by method II could retain the enzyme to a greater extent in both the supports. For further studies, immobilization was carried out by binding PI modified enzyme to the PEI treated supports (method II). The result for optimization of enzyme dosage for immobilization is presented in fig.3.

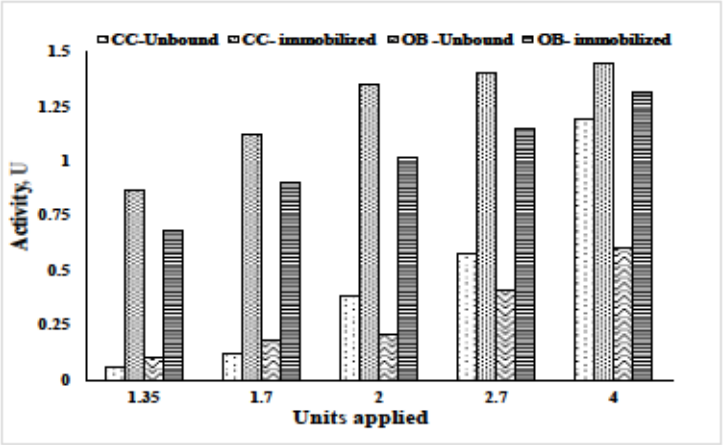

Figure 3 Optimization of enzyme dosage for immobilization of glucoamylase by method II

Based on these results, 2 units of PI treated enzyme was found to be the optimum load for cloth piece as maximal retention to an extent 67.5% occurred. Dosage of 1.68 units/ 0.2g of OB was optimal with a retention of 53% activity. It is well known that significant loss of activity occurs on covalent binding. The coupling efficiency and recovery of the enzyme by method II gave promising results. Enzyme immobilized by method II was characterized.

Characterization of the immobilized enzymes

The physico-chemical properties of the immobilized system were investigated and compared to those of free and PI treated forms. All the enzymes were optimally active at around the pH of 5.6-5.8. Immobilized enzymes however, exhibited broader pH-activity profiles as compared to those of native and PI oxidized forms (figure 4).

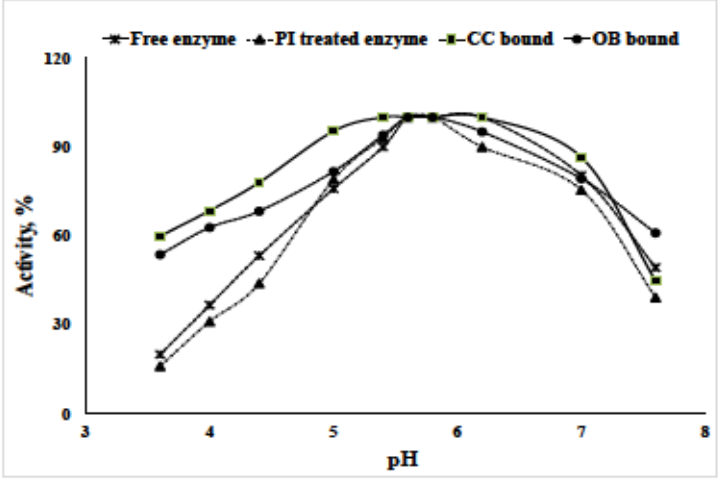

Figure 4 Effect of pH on the activity of glucoamylase preparations

The shift of pH and temperature profiles as a consequence of immobilization has been documented. Shift in pH/temperature-activity/stability profile or narrowing or broadening of the profiles as a consequence of immobilization have been reported (**Klibanov, 1983; Bachler *et al.*, 2004; Guzik *et al.*, 2014**). Both the immobilized forms were much more active at the acidic side of the pH optima. An enzyme attached to a poly electrolyte carrier may encounter micro-environmental effects in its immediate vicinity. Hydroxyl ions may accumulate at the poly-cationic carrier surface and hence the pH in the microenvironment of the enzyme is likely to be higher than the bulk solution. This may result in a shift in the pH versus activity profile to acidic side. Stability studies showed a significant degree of inactivation of free enzymes below pH 5 (fig 5A).

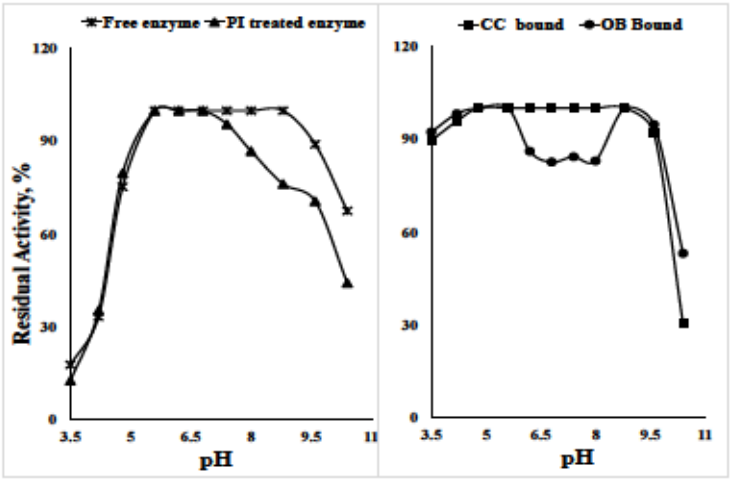

Figure 5 Effect of pH on the stability of the immobilized glucoamylase

The native enzyme which was stable in the range of pH 5.5- 8.8, lost its stability above pH 7 on oxidation with PI. The PI treated enzyme regained the stability in the alkaline side of pH and also attained stability in the acidic side of pH on immobilization to CC (fig 5b). Thus pH stability curve broadened on immobilization especially in case of enzyme coupled to CC. Enzyme immobilized on OB showed significant stabilization in acidic region. Interestingly, the residual activity was around 83-84% in phosphate buffer at pH 6.2 to 8.0. The stability increased to 100% at pH 8.8 and remained relatively stable till pH 9.6, decreasing thereafter. It appears that loss of activity at pH 6.2-8.0 may not be due to direct impact of pH on the stability of the enzyme. It was observed that the OB seeds swell further in size when incubated in phosphate buffers. This expansion might have had an impact on the structure of enzyme or the bond between the enzyme and support resulting in decreased activity. Effect of temperature on the activity of immobilized enzyme is exhibited in fig 6.

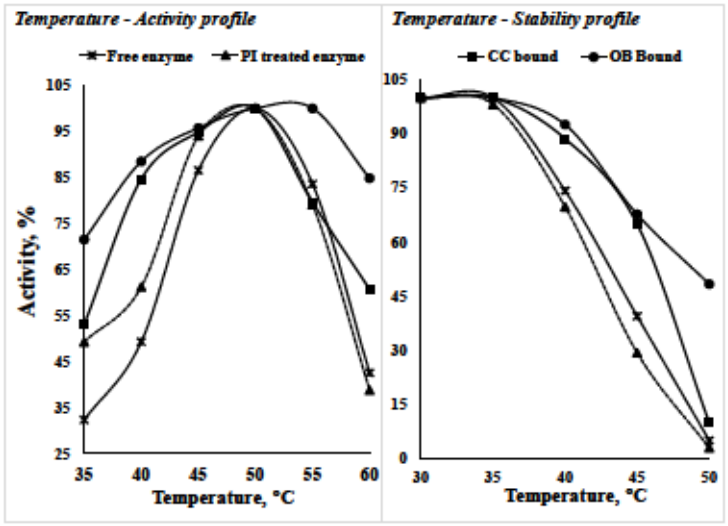

Figure 6 Effect of immobilization on the temperature-activity and thermo-stability profiles of the glucoamylase

The native enzyme, PI treated enzyme and both the immobilized forms were optimally active at 50°C. Enzyme immobilized on OB was optimally active at 50-55°C. Overall, both the immobilized forms exhibited relatively broader temperature zone of maximal activity and the free forms showed comparatively a narrow bell shaped curve. If unfolding is recognized as an indispensable step during thermal denaturation of an enzyme, then the more firm the protein moiety of the enzyme is fixed onto the support, the more difficult it is to unfold and inactivate the enzyme. Immobilization conferred protection to the enzymes against thermal denaturation to a significant extent as shown in figure 6.

The Km for native, PI treated enzyme, cloth bound and OB bound forms were 0.08, 0.105, 0.23 and 0.26 $mg.ml^{-1}$ respectively. The apparent increase in the Km values by 22-25 fold could be due to the limited diffusion of the substrates towards the enzyme active site and the movement of the formed products from the microenvironment of the enzyme to the bulk solution or, due to possible changes in the enzyme structure on being immobilized. Diffusional limitation may be more pronounced in case of high molecular mass molecules such as starch.

Starch saccharification studies

It was observed that the rate of starch hydrolysis was relatively linear up to 120min for both the bound forms when fed with 5 ml of 15 $mg.ml^{-1}$ starch. The immobilized systems were recycled 14 times (120min cycle each) over a period of 28h (figure.7).

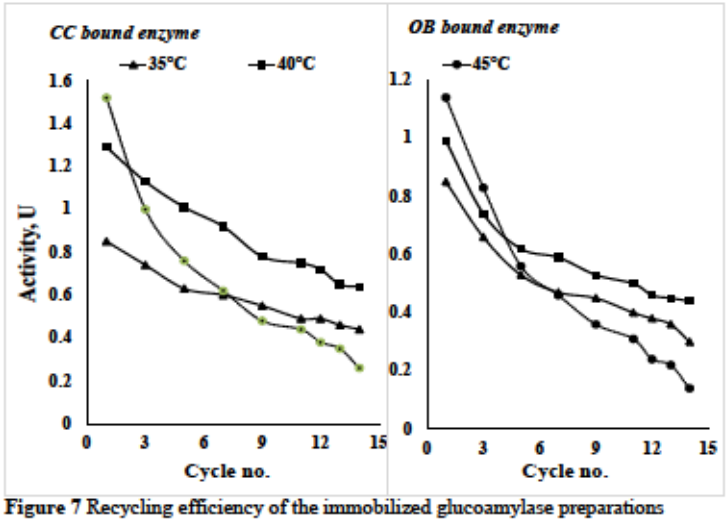

Figure 7 Recycling efficiency of the immobilized glucoamylase preparations

Enzyme coupled to CC could retain 50% of its activity at 40°C at the end of 13th cycle. Enzyme bound to OB seeds could retain 50% of its activity at 40°C at the end of 11th cycle. Both the systems lost 50% of their activity by the end of 5th cycle at 45°C. Total amount of glucose produced by immobilized cloth preparation in 14 cycles at 35°C, 40°C and 45°C were 182, 275 and 207mg respectively and 153, 187 and 155mg respectively by the OB bound enzyme. Product formation was maximum at 40°C for both the immobilized forms. Saccharification process was therefore carried out at the suboptimal temperature of 40°C (figure 8).

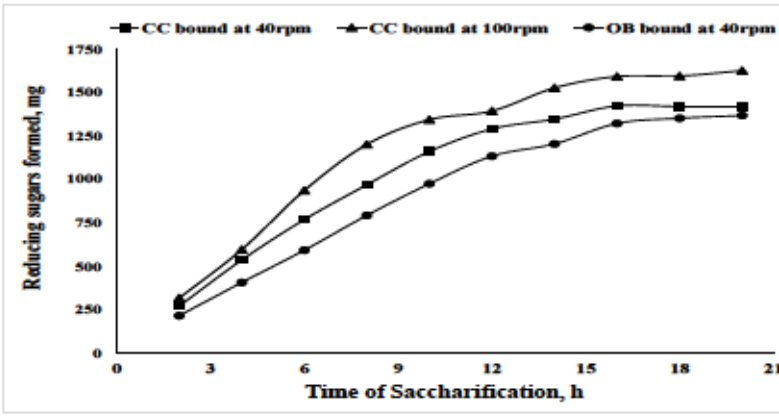

Figure 8 Continuous saccharification of starch by the immobilized preparations of glucoamylase

The enzymes in free state offered around 91.5-92% conversion. However, CC and OB bound preparations were able to convert starch only to an extent of 63.9 and 61.6% respectively at 40rpm. Decrease in % conversion of starch has been one of the major drawbacks suffered by most of the immobilized glucoamylases. As discussed earlier, affinity of the enzyme for the substrate decreased on immobilization. To assess the diffusional limitation as a factor influencing the apparent Km values, the immobilized enzyme was incubated with the substrate in a shaker water bath at 100 rpm to determine the Km. The Km values of 2.3 and 2.6 $mg.ml^{-1}$ (at 40rpm) decreased to 1.98 and 1.86 $mg.ml^{-1}$ for the enzyme coupled to cloth and OB seeds respectively. Diffusional limitation of the solutes, therefore appears to be one of the factors responsible for the apparent decrease in the affinity of the immobilized enzyme for starch. Functioning of CC bound preparation was relatively better than the OB preparation. The pellicular, mucilaginous coat of the OB seeds may undergo attrition at higher speeds of agitation when used continuously over long periods of time. Cloth does not encounter such issues. Saccharification of starch at 100rpm was therefore performed using cloth bound preparation. Saccharification to an extent of 73% could be achieved.

Both the enzyme preparations stored at 10°C (refrigeration) were stable upto 15 days. After 40 days of storage, the cloth bound enzyme lost 19.7% of its initial activity, whereas the OB bound seeds lost around 44% of activity.

CONCLUSION

Immobilization of the enzyme through its carbohydrate moieties appears to be a promising technique for immobilization of the glucoamylase produced by *A conoides* onto both the supports activated by treatment with PEI. Immobilization conferred stability to the enzyme in the acidic region, hinting at micro-environmental effect due to cationic nature of the PEI treated supports. Immobilised enzymes were relatively more thermostable than in its free state. Although the enzyme preparations were optimally active at 50°C, optimum temperature for saccharification (continuous use) of the enzyme was found to be 40°C. Km of the enzyme increased on immobilization. Starch being a high molecular weight substrate, the immobilized preparation appears to encounter diffusional limitation. Conversion of starch by CC bound preparation improved when saccharification was carried out at higher speed of agitation CC preparation may hence, be well suited for use in fluidized bed reactors. . The support chosen for immobilization must be low cost, easy to use, and easily available and must bind to the enzyme via a simple and inexpensive activation method. In addition, for use in food industries it has to be non-toxic, chemically inert (under conditions of use) and non-biodegradable (**Contesini *et al.*, 2013**) Cloth fulfils all these criteria and overall, cloth bound enzyme preparation was superior in its performance to the OB bound glucoamylase.

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AN EVALUATION OF ELISA USING RECOMBINANT P17 ANTIGEN FOR CATTLE BRUCELLOSIS

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ABSTRACT

Brucellae are Gram-negative cocccobacilli, facultative intracellular bacterial pathogens of both humans and animals. Brucellosis is an important disease that is difficult to diagnose and treat that causes heavy economic losses and human suffering. Diagnosis of brucellosis plays a vital role for control and prevention of the disease. Lipopolysaccharide (LPS) based Enzyme Linked Immuno Sorbent Assay (ELISA) shows false positivity due to cross-reactivity with other gram-negative bacteria LPS. The present investigation was undertaken to assess the diagnostic potential of the recombinant P17 protein of *Brucella*. P17 gene of *Brucella abortus* (*B. abortus*) was amplified, cloned and subcloned into pQE 30 vector yielding high levels of protein expression. The purified recombinant P17 (rP17) protein was used to develop an indirect ELISA (i-ELISA) test for brucellosis. The rP17-ELISA was compared with RBPT (Rose Bengal Precipitation Test) and LPS-ELISA using 530 cattle sera. The concordance percentage and kappa statistics of P17-ELISA is greater in compression with LPS-ELISA. Relative sensitivity and relative specificity of P17-ELISA shows a positive trend with RBPT. The data suggest that P17-ELISA can be a useful method for *Brucella* diagnosis and recombinant P17 protein is a potential antigen for diagnosis of cattle brucellosis.

Keywords: Brucellosis, Recombinant P17 antigen, ELISA, Cattle, RBPT, Diagnosis

INTRODUCTION

Brucella is a facultative intracellular zoonotic bacterium causing chronic disease resulting in a substantial economic loss worldwide (**Im *et al.*, 2016; Assenga *et al.*, 2015**). *Brucella* infection arises from occupational and domestic contact with infected animals or their discharges. Brucellosis causes abortion in female livestock's (cattle, sheep, and pigs) and in males; it causes orchitis (**Neta *et al.*, 2010**). Although *brucellosis* occurs throughout the world, it is most prevalent in the Middle East, Africa, Russia, India, South America, and Southern Europe and Latin American countries. Among all the species of *Brucella*, *B. abortus* causes brucellosis in cattle commonly, whereas cattle kept in close association with sheep or goats gets brucellosis by *B. melitensis* (**Lopes *et al.*, 2010**).
Twelve species of the genus *Brucella* have been identified using antigen (Ag) variation and primary host (**Sung *et al.*, 2014**). Diagnosis of brucellosis is based on serological tests like Standard Tube Agglutination Test (STAT), Complement Fixation Test (CFT), the Coombs test etc. were appropriate for all situations. For animal screening Herds Rose Bengal Plate Test (RBPT) and Buffered Plate Agglutination Test (BPAT) are more suitable. Molecular techniques like the Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) techniques are used to differentiate species and strains within the genus *Brucella* (**Yu *et al.*, 2010; Nielsen *et al.*, 1995**). The Enzyme-Linked Immunosorbent Assay (ELISA) can be used for screening and confirmation of brucellosis in one step while CFT is excellent in terms of sensitivity and specificity (**Nielsen *et al.*, 1995; Wright *et al.*, 1997**). Antibodies produced against *Brucella* smooth- Lipopolysaccharides (LPS) antigen, which cross-reacts with LPS of *E. coli* O157 (**Stuart *et al*, 1982**), *Stenotrophomonas maltophilia* (**Corbel *et al.*, 1984**), and *Yersina enterocolitica* O:9, leading to false positivity. Various diagnostic proteins of *Brucella* has been known, which includes the 89 kDa protein, Outer membrane proteins (OMPs) like OMP36, 28 , 16, 10, Cell surface proteins like 31.6, 32.5, 58.5 and 14.7 kDa (**Kaushik *et al.*, 2009**), periplasmic protein BP26 (Kumar *et al* ., 2008) and cytoplasmic proteins P39, P17 and P15 . P17 is a cytoplasmic protein and thought to be antigenic in nature and also a potential candidate for the diagnostic purpose (**Letesson *et al.*, 1997; Hemmen *et al.*, 1995; Büyüktanir et *al.*, 2011**). The present work has been envisaged to express the P17 gene of *B. abortus* and to evaluate the diagnostic potential of recombinantP17 protein using indirect ELISA.

MATERIALS AND METHOD

Bacterial Strains, Growth Conditions, and Vectors

Escherichia coli DH5α as the host and pTZ57R/T cloning vector (Thermo Scientific, Fermentas) was used as the cloning vector for the cloning of the gene encodes P17 derived from genomic DNA of *B. abortus* (S19) from Indian Veterinary Research Institute (IVRI), izatnagar, India. The protein expression vector was pQE30 (Qiagen, USA), and *E. coli* Novablue (Novagen) strain was used as the screening host for pQE30 bearing inserts. Bacterial strain for expression of recombinant protein was *E. coli* BL21 (Novagen). All the *E. coli* bacterial cultures were grown at 37°C on Luria–Bertani (LB) agar plates or in LB medium. Where appropriate, media was amended with various substrates and 100 µg ampicillin/ml.

PCR amplification and cloning P17 F and P17 R

B. abortus strain (19) gene (which will encodes outer membrane protein MWt 17 kDa) was amplified by PCR, using a set of primers P17 F and P17 R (5'- CGG GGATCC ATGAACACTCTGGCTAGCAAT-3' and 5-CGGC AAGCTT TTACTTGATTTCAAAAACGAC-3') designed from the available nucleotide sequence accession number DQ437516.1. A high fidelity Novagen KOD XL DNA polymerase was used for DNA amplification, which was carried out with 30 cycles of denaturation (60 s at 94 °C), annealing (1 min at 55 °C) and extension (1 min at 72 °C), followed by 10 min of further extension at 72 °C. The PCR amplified product was separated by agarose gel, purified by gel elution and ligated into the pTZ57R/T cloning vector according to the manufacturer's instructions and then transformed into *E. coli* DH5α. The pTZ57R/T vector carrying the P17 gene was digested with the enzyme Bam H1 and Sal I, and the produced fragment was gel-purified before subcloning into an expression vector. The target DNA fragment was sub-cloned at the Bam H1 and Sal I site of pQE30vector, resulting in pQEP17. The vectors thus obtained were transformed into *E. coli* BL21 for fusion protein expression.

Induction, purification, and immunoblot assays of recombinant protein

E. coli cells harboring pQEP17 plasmid were grown in Luria-Bertani (LB) medium till the OD600nm reached to 0.5. The cells were then induced with 1 mM IPTG and allowed to grow further for 6h at 37^{0}C. Cells were harvested and analyzed using Sodium dodecyl sulfate Polyacrylamide gel electrophoresis (SDS-PAGE). Ni-NTA agarose purified protein was analyzed on SDS-PAGE and transferred to nitrocellulose membranes in a semi-dry transblot system (Atto, Japan). Transferred proteins were immunostained with field sera at a dilution of 1:200. Secondary antibodies conjugated to horseradish peroxidase (Sigma) and were used in the assays. The reactions were developed with Diamino Benzidine (Sigma) and Hydrogen peroxide (H_2O_2).

Bioinformatic analysis

The obtained DNA sequence was translated in EMBL database and obtained protein was analyzed in protean of DNASTAR.7.0.

Extraction and quantification of LPS

Extraction of *B. abortus* S19 Smooth-LPS was extracted by the hot phenol-water method. In brief, 50g wet weight of cells was suspended in 170ml of distilled water, followed by the addition of 190ml of 90% (v/v) hot phenol (66°C). After 30mins, the mixture was centrifuged and phenol layer was removed. The LPS in the resultant mixture was precipitated by cold methanol (4°C) and dissolved in 0.1M tris buffer. Proteinase K (50µg per 10mg protein) and then both DNase and RNase (50µg per 1mg nucleic acid) added to extracted samples to reduce protein and nucleic acid contaminations. The antigen was run in SDS-PAGE and stained using silver staining followed by quantification using the phenol- sulphuric acid method in microplate format (**Salmani et *al.*, 2008**).

Recombinant p17- ELISA

Clinical sera from cattle were analyzed by indirect ELISA using recombinant P17 as test antigen. The immunoassay plates (Maxisorp, Nunc, Denmark) were coated with purified recombinant P17 protein at a concentration of 100 ng per well, diluted in 0.1 M bicarbonate buffer (pH 9.0) and incubated at 4 ^{0}C, overnight. The wells were washed five times with phosphate buffer saline-Tween20 (PBST) and then blocked with 5% BSA. Immunoassay plates were charged with sera at a dilution of 1:100 and incubated at 37^{0}C for 1 h. After washing with PBST for five times the plates were incubated with HRPO conjugates for 1 h at 37^{0}C. After washing with PBST, the wells of immunoassay plates were fill with a substrate solution containing Ortho-phenyl diamine (OPD) and H2O2. Colour development was stopped by adding 2 M H2SO4, after 10 min of incubation of the plates in dark at room temperature. Absorbance was recorded at 490 nm wavelength in an ELISA reader.

Rose Bengal plate agglutination test

The RBPT was performed on a glass plate using 30µl of antigen and 30 µl of serum. Both serum and antigen are mixed thoroughly. Any degree of agglutination within 3 min was taken as positive. RBPT antigen was procured from Division of Biological products, Indian Veterinary Research Institute (IVRI), Izatnagar.

Evaluation of RBPT and rP17ELISA, and rP17 ELISA and LPS ELISA

The relative sensitivity, specificity, Positive predictive value (PPV), Negative predictive value (NPV) and accuracy of recombinant P17 ELISA for serodiagnosis of brucellosis were evaluated in comparison to RBPT as well as LPS ELISA as described below (**Chaudhuri *et al.*, 2010**).,

$$\text{Relative sensitivity} = a/(a+c) \times 100$$

$$\text{Relative specicity} = d/(b+d) \times 100$$

$$\text{Accuracy} = (a+d)/(a+b+c) \times 100$$

$$\text{PPV} = a/(a+b)$$

$$\text{NPV} = d/(c+d)$$

$$\text{Apparent prevalence} = a+b/N \times 100$$

$$\text{True prevalence} = a+c/N \times 100$$

$$\text{Accuracy} = a+d/N \times 100, \text{ where}$$

'a' is the number of sera positive by ELISA and RBPT
'b' is the number of sera negative by RBPT but positive by ELISA
'c' is the number of sera positive by RBPT but negative by ELISA
'd' is the number of sera negative by ELISA and RBPT
'N' is the total number of samples (a + b + c + d)

Statistical analysis of RBPT and ELISA

Concordance and Kappa statistics of rP17ELISA with a well as LPS ELISA were assessed as described as follows (**Gwida *et al.*, 2011**)

RESULTS

Construction and expression of P17 Gene.

The P17 gene was successfully amplified from the *B. abortus* S19 genomic DNA. The obtained PCR product of 442 bp was cloned to the and pTZ57R/T cloning vector for DNA sequence analysis and then *Bam* HI and *Sal* I digested P17 fragment was subcloned at *Bam* HI and *Sal* I cleaved pQE30 expression vector. In this recombinant plasmid, the P17 sequence was fused with 6x His tag and was tightly regulated by a T7 promoter. All constructs were confirmed by restriction analysis and DNA sequencing. The nucleotide sequence reported in this paper has been deposited in the GenBank DNA databases with the Genbank Id: KJ702467.1. The resulting clone was named pQEP17 recombinant plasmid.

Analysis of recombinant P17 protein by SDS-PAGE and Western Blot

E. coli cells harboring the recombinant plasmid pQEP17 was induced with 1M IPTG for overnight produced a predominant band similar to 17kDa protein on 15% SDS-PAGE (Fig 1, Lane-2). No such protein band was observed in uninduced *E. coli* cells harboring recombinant plasmid pQEP17 (Fig 1, Lane-1). Histidine-tagged expressed recombinant protein was purified using Ni-NTA beads (affinity chromatography) produced a single band of 17kDa protein on SDS-PAGE (Fig 1, Lane-3).
The Polyclonal serum was raised in rabbits, by injecting the purified rp17 protein with 2 to 3 boosters. Polyclonal serum against rP17 protein could bind to expressed pQEP17 protein (Fig 2, Lane-2), as well as purified E. coli, expressed 17kDa protein (Fig 2, Lane-1), as detected by Western blot analysis.

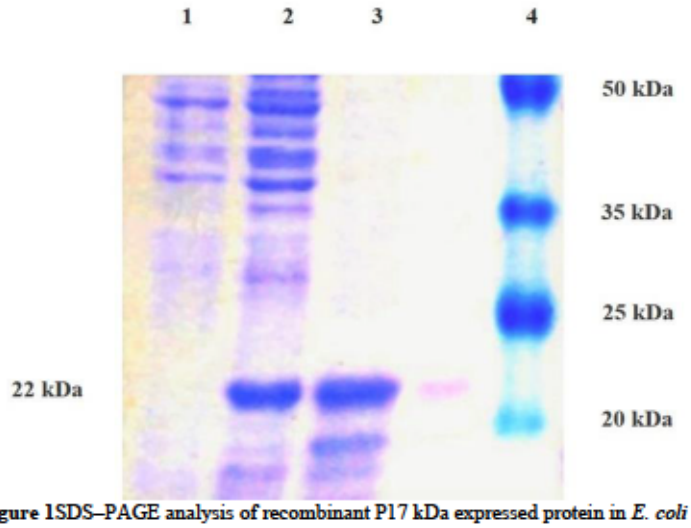

Figure 1SDS–PAGE analysis of recombinant P17 kDa expressed protein in *E. coli*
Lane 1, Uninduced *E. coli* cells containing recombinant pQEP17 insert
Lane 2, Induced *E. coli* cells containing recombinant pQEP17 insert
Lane 3, Purified P17 kDa protein
Lane 4, Pre-stained protein Marker

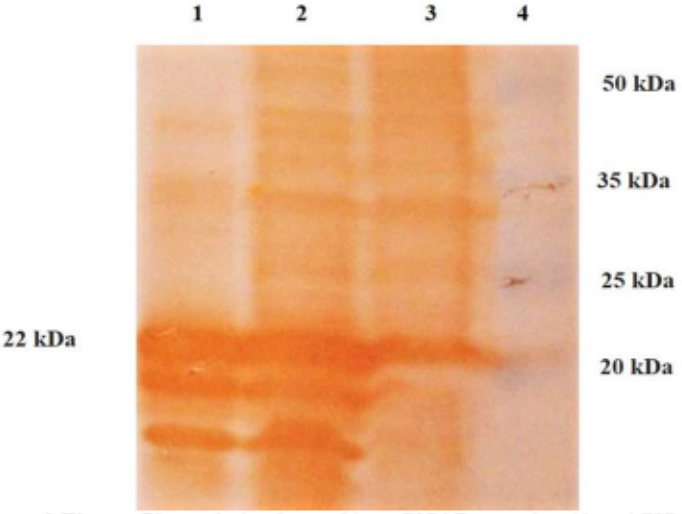

Figure 2 Western Blot analysis of recombinant P17 kDa protein with anti-P17 specific serum
Lane 1, Purified P17 kDa protein
Lane 2, Induced *E. coli* cells containing recombinant pQEP17 insert
Lane 3, Uninduced *E. coli* cells containing recombinant pQEP17 insert
Lane 4, Pre-stained protein Marker

rP17Elisa standardization and comparison with RBPT, LPS ELISA

The recombinant protein and LPS were used at the concentration of 62.5 ng and 125 ng respectively per well, and at a serum dilution of 1:100, the test got standardized (**Tiwari *et al.*, 2013**). Twenty known positives and twenty-five known negative samples were taken as gold standard and recombinant P17-ELISA (rP17-ELISA) and LPS-ELISA was performed (Table 1). The mean value of sera at OD490nm + 3 SD (standard deviation) was considered as cut-off value for declaring a serum as positive or negative for brucellosis. The ELISA test results of rP17, RBPT, LPS were shown same results with gold standard samples indicates that the rP17 ELISA is accurate in compression with the other methods.

Table 1 Comparison of rP17 ELISA with RBPT and LPS ELISA

	Known Positives (20 nos)	Known Negative (25 nos)
rP17 ELISA	20	25
RBPT ELISA	20	25
LPS ELISA	20	25

Evaluation of RBPT and rP17ELISA, and rP17 ELISA and LPS ELISA

The study has been conducted with 530 unknown samples to evaluate the rP17 ELISA sensitivity and specificity with the RBPT and LPS ELISA. The rP17-ELISA results showed (86.06%) relative sensitivity and (93.62%) relative specificity on comparison with RBPT (Table 2). Negative predictive value was (95.02%), while positive predictive value was (80.15%) (Table 2) between RBPT and rP17ELISA. The accuracy of prediction was 91.88% (Table 2) between RBPT and rP17-ELISA. rP17-ELISA showed (84.28%) relative sensitivity and (93.60%) on comparison with LPS-ELISA (Table 3). The accuracy of prediction was 91.32% between rP17-ELISA and LPS-ELISA. Negative predictive value was (94.32%), while positive predictive value was (83.09%) (Table 3). RBPT and LPS- ELISA showed the sensitivity of (84.28%) and specificity of (93.60%). (Table 4)

Table 2 Comparison of RBPT and P17-ELISA in diagnosis of cattle brucellosis

Serological test		RBPT		
		Positive	Negative	Total
P17-ELISA	Positive	105 (a)	25 (b)	130 (a+b)
	Negative	20 (c)	380 (d)	400 (c+d)

Relative Sensitivity =a / a+c×100=86.06%
Relative Specificity=d / b+d×100=93.62%
Positive Predictivevalue=a / a+b×100=80.15%
Negative Predictivevalue=d / c+d×100=95.02%
Apparent Prevalence=a+b / N×100=24.71%
True Prevalence=a+c / N×100=23.01%
Accuracy of prediction=a+d / N×=91.88%

Table 3 Comparison of LPS-ELISA and P17-ELISA in diagnosis of cattle brucellosis

Serological test		LPS-ELISA		
		Positive	Negative	Total
P17-ELISA	Positive	118 (a)	24 (b)	142 (a+b)
	Negative	22(c)	366 (d)	388(c+d)

Relative Sensitivity =a / a+c×100= 84.28%
Relative Specificity=d / b+d×100=93.60%
Positive Predictivevalue=a / a+b×100=83.09%
Negative Predictivevalue=d / c+d×100=94.32%
Apparent Prevalence=a+b / N×100=26.79%
True Prevalence=a+c / N×100=23.41%
Accuracy of prediction=a+d / N×=91.32%

Table 4 Comparison of RBPT and LPS -ELISA in diagnosis of cattle brucellosis

Serological test		RBPT		
		Positive	Negative	Total
LPS-ELISA	Positive	113(a)	22(b)	135 (a+b)
	Negative	6(c)	389(d)	395 (c+d)

Relative Sensitivity =a / a+c×100= 94.95%
Relative Specificity=d / b+d×100=94.64%
Positive Predictivevalue=a / a+b×100=94.95%
Negative Predictivevalue=d / c+d×100=98.48%
Apparent Prevalence=a+b / N×100=25.47%
True Prevalence=a+c / N×100=22.45%
Accuracy of prediction=a+d / N×=94.71%

Statistical analysis of RBPT and ELISA'S

Concordance was maximum between P17-ELISA and LPS- ELISA, which was (94.71%), followed by RBPT and P17-ELISA test with a concordance of (91.40%). Minimum concordance of (91.32%) was observed between RBPT and LPS-ELISA. In Kappa statistics, LPS- ELISA and P17-ELISA showed almost perfect agreement as the value observed was .833. (Table 4)

Table 4 Concordance and Kappa statistics between different tests for cattle brucellosis

Test 1	Test 2	Concordance (%)	Kappa value
RBPT	**P17-ELISA**	91.40	0.778
RBPT	**LPS-ELISA**	91.32	0.871
P17-ELISA	**LPS-ELISA**	94.71	0.833

DISCUSSION

Brucella diagnosis is based on the detection of antibodies generated against immunodominant antigenic molecules using serological methods. The RBPT is the commonly used test at field levels, based on the agglutination of antibodies. Although RBPT sensitivity is high but has low specificity also. *E. coli* O: 159, *Y. enterocolitica* O: *Vibrio cholera,* and *Salmonella sp.* show cross-reactivity with other clinically important bacterias leading to false positive results in various tests (**Im *et al.*, 2016**). Different ELISA models and FPA have been developed using LPS and O-polysaccharide as diagnostic antigen (**Nielsen *et al.*, 1997; Genc *et al.*, 2011; Saegerman *et al.*, 2004; Munoz *et al.*, 2005**). Similar epitope sharing between LPS of *Brucella* and that of other Gram-negative bacteria is the major cause of cross-reactivity in brucellosis infection (**Kaltungo *et al.*, 2014; Godfroid ., 2010**).

To overcome this problem, efforts have been made to improve the serodiagnosis of brucellosis by replacing the native antigens with highly purified specific recombinant antigens. It is expected that the recombinant protein will have less cross-reactivity and might act as better potential diagnostic antigens in animal brucellosis (**Thavaselvam, *et al.*, 2010**). The recombinant-based serological test showed high sensitivity and specificity owing to the high concentration of immunoreactive antigens and lack of non-specific molecules present in the whole cell preparation. Few cytoplasmic Ags of *Brucella* P17, P15, and P19 has shown to be antigenic in nature (**Letesson *et al.*, 1997**). Hence, in the present study, the rP17 protein was expressed, purified and rP17 ELISA was standardized and compared with LPS ELISA and RBPT. DNA encoding the P17 protein was cloned, sequenced and the amino acid sequence of rP17 was deduced. The obtained accession number is KJ702467.1. The blast results in NCBI matched with lumazine synthase of *Brucella*, a cytoplasmic protein (**Berguer *et al.*, 2012**). Tthe sequence was analyzed using Protean in DNA Star program showed that the protein has 6.933 isoelectric Point and negatively charged at pH-7.0. The P17 protein had both 4 alpha helix and 1 beta regions, the alpha region could play an important role in antigenicity. Molecular Weight of the protein was predicted as 17354.92 Daltons containing 158 amino acids. The protein had 15 Strongly Basic(+) Amino Acids (K,R), 17 Strongly Acidic(-) Amino Acids (D,E), 71 Hydrophobic Amino Acids (A,I,L,F,W,V) and 27 Polar Amino Acids (N,C,Q,S,T,Y) (**Goldbaum *et al* 1999; Bagath *et al.*, 2015**).

The P17 protein was expressed in the form of inclusion bodies, which is a common feature when proteins are expressed at very high level. Nickel –NTA agarose resin, was used to purify the expressed P17 protein having Histidine tag at the N-terminal end which is similar to other recombinant *Brucella* protein

purification (**Letesson *et al.*, 1997**). Reactivity of cattle sera with rP17 in western blotting indicated the similarity of reports published by Hemmen *et al.*, and Letesson *et al.*, in the year 1995 and 1997 respectively. ELISA is more sensitive than conventional tests, hence it is regarded as meritorious over RBPT in the diagnosis of brucellosis. However, RBPT has less specificity leading to false positivity (**Chaudhuri *et al.*, 2010**). Moreover, ELISA assays were reported to be more sensitive, to give positive results sooner after infection in non-vaccinated animals and to be more persistent than the traditional serological tests in both experimentally and naturally infected animals (**Saegerman *et al.*, 2004**).

Comparison of rP17-ELISA with LPS-ELISA and RBPT, rP17-ELISA showed (24.52%) seropositivity while LPS-ELISA showed (27.73%) seropositivity, for a total of 530 sera samples. The reason why LPS-ELISA showed more seropositivity is due to the presence of high cross-reacting antibody produced against LPS with other gram-negative bacteria (**Tiwari *et al.*, 2011**). RBPT also showed more seropositivity when compared with rP17-ELISA, due to the use of the whole cell antigen for agglutination reaction (**Tiwari *et al.*, 2011**). The RBPT should be used in combination with iELISA as rapid, simple and easy methods for screening (**Ahmed *et al.*, 2016**). Concordance between rP17-ELISA and RBPT was higher than concordance between LPS-ELISA and RBPT. In laboratories, the iELISA for brucellosis diagnosis could also be used as a single diagnostic test, where the ELISA technique is already used for the diagnosis of other diseases. The agreement between rP17-ELISA and LPS-ELISA was significant, as determined by the Kappa index of concordance of 0.83 between tests, a similar finding was observed by Gwida in the year 2011. Further, P17 coated in the ELISA plates and was able to react even after 6 months of storage at 4^{0}C, which showed the stability of the protein to sustain for a longer period of time at lower temperature. In the present study, we have used 17 kDa proteins (P17) of *B. abortus* strain (19) for identification of anti-*Brucella* antibodies in the sera collected from different parts of the country. Our result was in accordance with previous reports describing the usefulness of this protein for detecting anti-*Brucella* antibodies to cattle (**Kumar *et al.*, 2008**). Both the Kappa value and the Concordance percentage of rP17-ELISA with LPS-ELISA were almost perfect and had maximum agreement respectively. Relative sensitivity and relative specificity of rP17-ELISA was 86.06 % and 93.62%, respectively, when compared with LPS-ELISA. Relative sensitivity and relative specificity of P17-ELISA was also on a positive trend with RBPT.

In conclusion, it can be inferred that the recombinant P17 protein was successfully expressed in *E. coli*. The yield of recombinant P17 protein was high, which will make the test cost effective. The tendency of the protein to remain stable for months makes P17 a suitable candidate in the field. Both Kappa value and concordance percentage of P17-ELISA with LPS-ELISA were perfect and had maximum agreement respectively. Relative sensitivity and relative specificity of P17-ELISA was 86.06 % and 93.62% respectively when compared with LPS-ELISA. Based on the results obtained, it can be concluded that recombinant P17 protein is a potential candidate antigen for serodiagnosis of cattle brucellosis.

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REVISITING MOLECULAR CLONING TO SOLVE GENOME SEQUENCING PROJECT CONFLICTS

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ABSTRACT

In our laboratory, DNA sequencing by Sanger method is used as the "gold standard" for clinical diagnostics, microbe identification (bacteria and yeast, mainly) and genome characterization. In this research, we used it to characterize a conflicting locus in a *Saccharomyces cerevisiae* sequencing project. When sequenced, the resulting electropherogram of the analyzed locus showed a pattern indicating either sample contamination or allele variation. Molecular cloning was chosen as the most straight-forward strategy to solve the dilemma. The initial characterization of recombinant plasmids by restriction enzyme digestion confirmed the presence of two genomic sequences. Their Sanger sequencing revealed two alleles distinguishable by a total of 29 nucleotide differences (25 of which were SNPs). NCBI BLAST revealed that the conflicting locus covered an intergenic region and a coding sequence for a putative permease protein. The present study shows the utility of the classical molecular cloning technique to solve problems of modern genome projects.

Keywords: Allele segregation, molecular cloning, Sanger sequencing, troubleshooting

INTRODUCTION

Molecular cloning is a methodology conformed by a set of experiments that allow the asexual exponential copying of an isolated genomic region for posterior analyses and genetic manipulation (**Sambrook and Russell, 2001**). The principle of this methodology relies on the use of plasmid vectors, restriction enzymes, ligases, calcium-induced bacterial transformation, and clonal propagation of recombined microorganisms (*Escherichia coli* and *Saccharomyces cerevisiae*, among others). This clonal propagation provides an additional value of this tool since allows segregating and thus the identification of coexisting highly similar gene sequences (**Barrera, Seeburg and Saunders, 1983**). Molecular cloning was eventually substituted by the development of the Polymerase Chain Reaction (PCR) in which there is no need for a host cell, and exponential multiplication of DNA material can be done *in vitro* and in a much shorter time (**K. B. Mullis, *et al.* 1986**).

Molecular cloning coupled to nucleotide sequencing has been commonly used to read genomes represented in clones of genomic libraries. In our case, the main objective was to achieve the resolution of a possible mix of genomes represented in a PCR product labeled Sc790Gap1, derived from the *S. cerevisiae* strain 790 genome, using molecular cloning to solve the putative overlapping sequences of at least two genetic variants.

MATERIAL AND METHODS

Conflicting amplicon

Gap1 is one of various genomic regions present in *S. cerevisiae* strain 790, amplified with *Gap1F* and *Gap1R* primers, troubling the genome assembly of this strain. Its sequences were obtained using the Applied Biosystems® (AB) Genetic Analyzer 3130 (AB, Foster city, CA, USA) while its analyses were attempted with various bioinformatics tools: Clone Manager 6 (Scientific Educational Software; NC, USA), SnapGene Viewer 2.6.2 (GSL Biotech LLC, IL, USA), BioEdit 7.2.5 (Biosciences, CA, USA) and MEGA 6.06 (**Tamura, *et al.*, 2013**).

DNA preparation

The yeast strain was harvested from an axenic culture used for beer production, and its total DNA was isolated as previously described by **Harju, *et al.*, (2004)**.

PCR amplification

PCR reaction was prepared using *Gap* primers (*Gap1F*: 5'-TTTACCATGAGCGCAACAGC-3' and *Gap1R:* 5'-AAAAAGCAGAACGACGCACC-3') at 0.6 µM, 100 ng of DNA sample, 11 µl of Master Mix (2X) from Promega (Madison, WI, USA), and nuclease free water (NF H_2O) to complete 15 µl. All amplification reactions were hot started at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 60s, annealing at 58°C for 60s, and extension at 72°C for 120s. After a final extension step at 72°C for 3 min, the amplification product was stored at 4°C.

Sequencing of amplicons

The PCR products were treated with Shrimp Alkaline Phosphatase (SAP) and Exonuclease I (EXO I) (Affymetrix, Santa Clara, CA, USA) enzymes during 15 minutes at 37°C, followed by 15 minutes at 80°C. 1 µl of treated product was added to a PCR sequencing master mix with 2 µl of Big Dye (Applied Biosystems, Foster city, CA, USA), 0.5 pmol of primer and pure sterile water up to 6 µl. The resulting PCR product was purified again with the SAM/X-terminator Kit (Applied Biosystems). The supernatant was loaded into the AB Genetic Analyzer 3130 for sequencing. Finally, raw data was processed using the Sequencing Analysis Software v5.3.1 (Applied Biosystems) to obtain the electropherograms.

Molecular cloning

The PCR product was cloned into a TOPO XL plasmid (Invitrogen, Carlsbad, CA) using the T/A cloning approach (**Sambrook and Russell, 2001**). Bacteria were transformed using heat shock and a rapid screening of the clones was performed through colony PCR using *Gap* primers (**Sambrook and Russell, 2001**). Lastly, a restriction analysis of the PCR products was realized using *TaqI* enzyme (New England Biolabs, Ipswich, MA) during one hour at 65°C in order to select the clones with a differential pattern.

Plasmid screening and characterization

Selected colonies were picked up with sterile toothpicks, inoculated in 3 mL of LB (Luria-Bertani) broth and incubated overnight at 37°C. Biomass was collected by microfugation and plasmids were isolated essentially as described by **Birboim and Doly, (1979)**. Insert presence and orientation were established by using restriction mapping and positive clones were sequenced using Sanger sequencing (**Smith, *et al.*, 1986**).

Sequence analyses

Sequences were aligned against consensus sequences found in GenBank using Bioinformatic software MEGA (**Tamura, *et al.*, 2013**).

RESULTS AND DISCUSSION

Sanger sequencing

The product obtained from the PCR reaction was bidirectionally read using *Gap1F* and *Gap1R* primers (according to the PCR assay, an expected product of approximately 1750 bp was seen). A clear reading of the first ~200 bp was obtained from the *Gap1F* primer; whereas approximately 400 bp were obtained using *Gap1R* primer. Beyond these lengths, electropherogram anomalies complicated its interpretation. Apparently, the artifact detected was due to two overlapping readings, and troubleshooting advice suggested that this could be due either to contamination or heteroploidy. Nevertheless, seeking to discard possible errors or artifacts, we designed new nested primers (*IntergapF* and *IntergapR*) located closer to the troubling region (Figure 1). Again, the same undecipherable readings were obtained (data not shown). Therefore, we decided to test the heterozygosity hypothesis and for this, molecular cloning was chosen as the most suitable method to segregate alleles in order to characterize them separately.

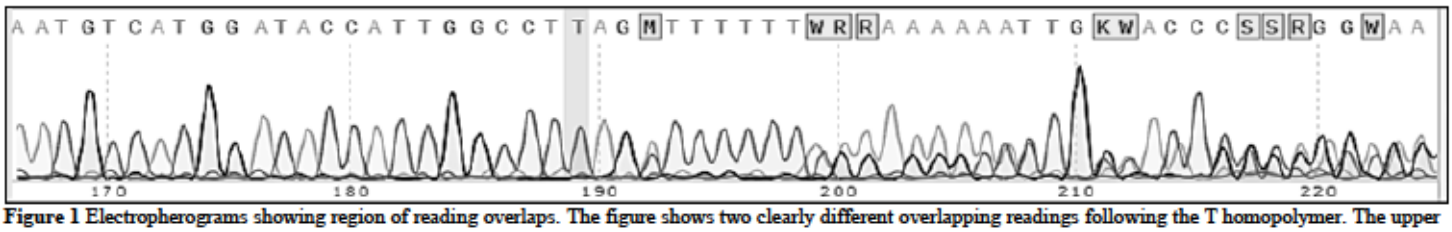

Figure 1 Electropherograms showing region of reading overlaps. The figure shows two clearly different overlapping readings following the T homopolymer. The upper sequence belongs to the forward end reading using *Gap1F* primer (5'>3'), while the lower one corresponds to the reverse end obtained with *Gap1R* primer (3'>5').

Molecular cloning and resolving sequencing

The original PCR product was inserted into a plasmid vector and then used for transforming *E. coli* cells to generate clones and continue with their identification, independent propagation and characterization of the cloned insert. The presence of the alleles in the transformed candidate clones was confirmed by restriction analysis using *Taq*I restriction enzyme of products obtained by colony PCR. Two different patterns predicted *in silico* were evidenced in the agarose gel image (Figure 2) and their insert orientation was established by double digestion with *Bgl*II and *Kpn*I enzymes (Figure 3).

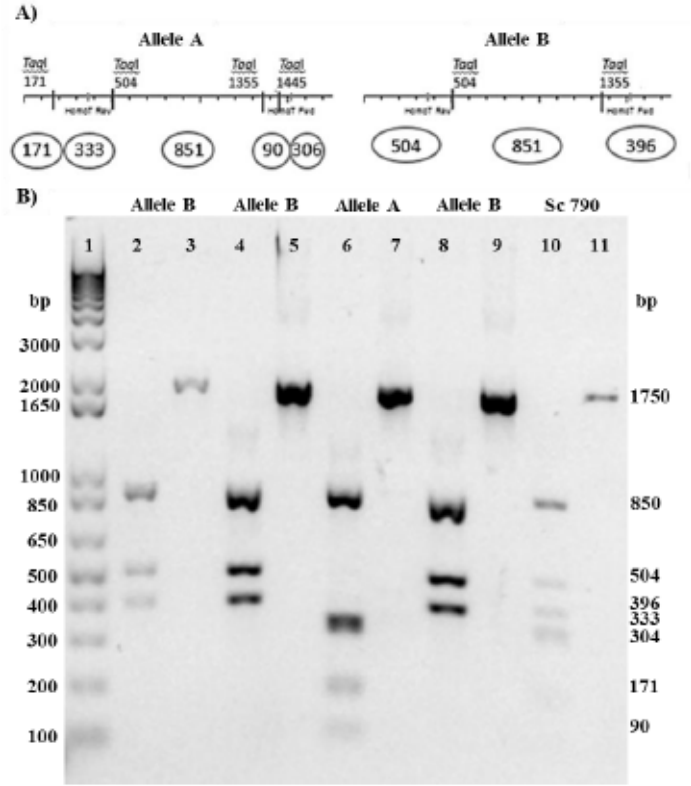

Figure 2 Characterization of clones by PCR and restriction analyses. (A) *In silico* *Taq*I restriction maps of possible PCR products obtained from recombinant plasmids using *Gap1F* and *Gap1R* primers. (B) Agarose gel electrophoresis of actual digestions with *Taq*I of the PCR products. Lane 1 shows the DNA base pair (bp) ladder. Lanes 2, 4 and 8 correspond to the size pattern identified as "Allele B". Lane 6 shows the pattern characterizing "Allele A". Lane 10 shows the restriction of the PCR product obtained directly from *S. cerevisiae* 790 (GCF_000146045.2), and lanes 3, 5, 7, 9, and 11 correspond to PCR products from the recombinant clones analyzed.

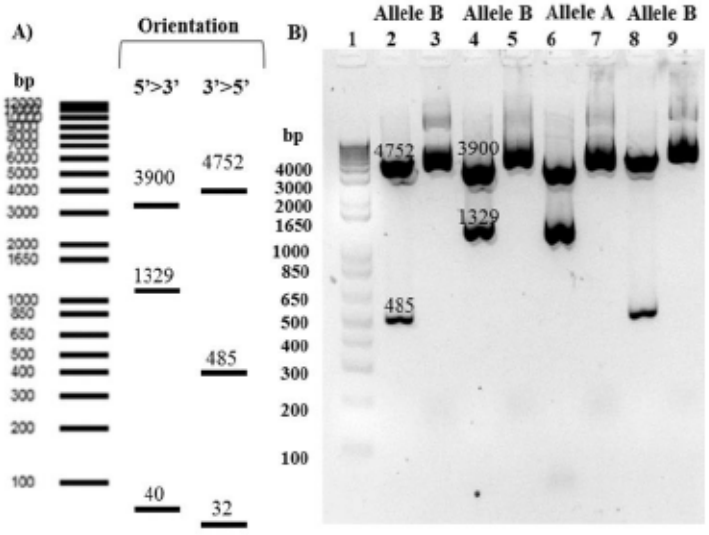

Figure 3 Strategy to determine the insert orientation in the plasmid. (A) *In silico* prediction of the two possible digestion patterns using *Bgl*II y *Kpn*I enzymes to determine the orientation of the cloned amplicon inserted into the plasmid. (B) Agarose gel electrophoresis showing the experimental results from the digestion assay. Lane 1 shows the DNA bp ladder. Lanes 2 and 8 show the pattern for insertion in the 3'>5'orientation, whereas lanes 4 and 6 present the opposite orientation pattern. Lanes 3, 5, 7, and 9 correspond to undigested plasmid DNA.

The cloning segregation (Fig. 3B, clones in lanes 4 and 6) permitted the sequencing of the two different alleles (resulting in the reading of sequences of approximately 850 nucleotide bases of good quality from each end), detecting four indel positions throughout T homopolymers, which were probably the primary cause of electropherograms overlapping. In addition, 25 single nucleotide polymorphisms (SNPs) were identified (Figure 4).

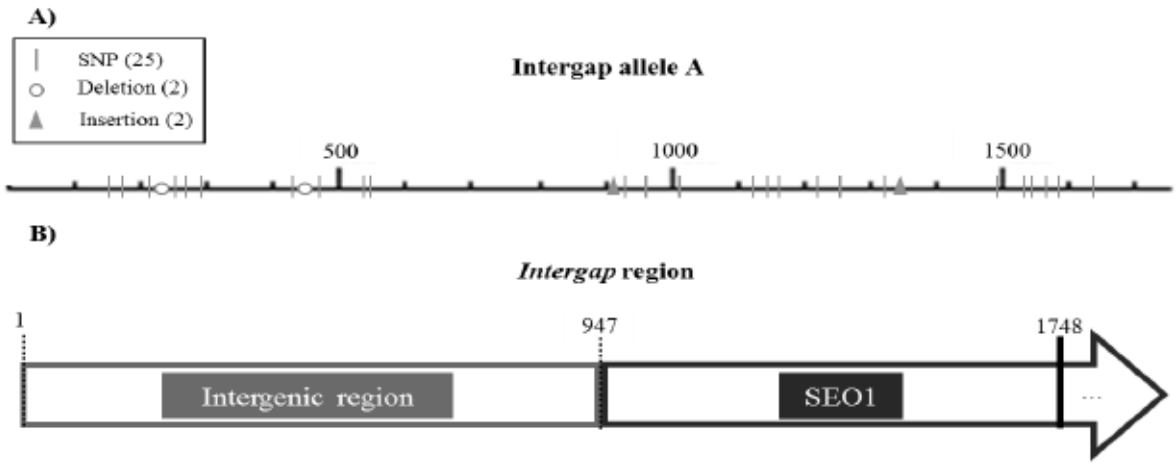

Figure 4 Genetic elements contained in the *Gap1* region. (A) Schematic representation of sequence features of allele A with respect to allele B sequences. *Vertical bars* indicate SNPs (25), *circles* indicate deletions and *triangles* represent insertions. (B) The alleles sequences cover an intergenic region (1-946 bp) and the starting coding region (947-1748 bp) of an open reading frame (a permease protein called SEO1) for a total length of 1748 bp.

Having solved the *Gap* region sequencing problem, its correct genome assembly and annotation was achieved. It turned out that the locus codes for a putative permease (SEO1, NM_001178208) and an intergenic region (Figure 5). The genome variants show a 98.51% similarity, and their origin was traced back to strains EC1118 (**Novo, *et al.*, 2009**) and YJM1463 (**Strope, *et al.*,2015**) of *Saccharomyces* using the GenBank database (**Benson, *et al.*, 2005**) (Table 1).

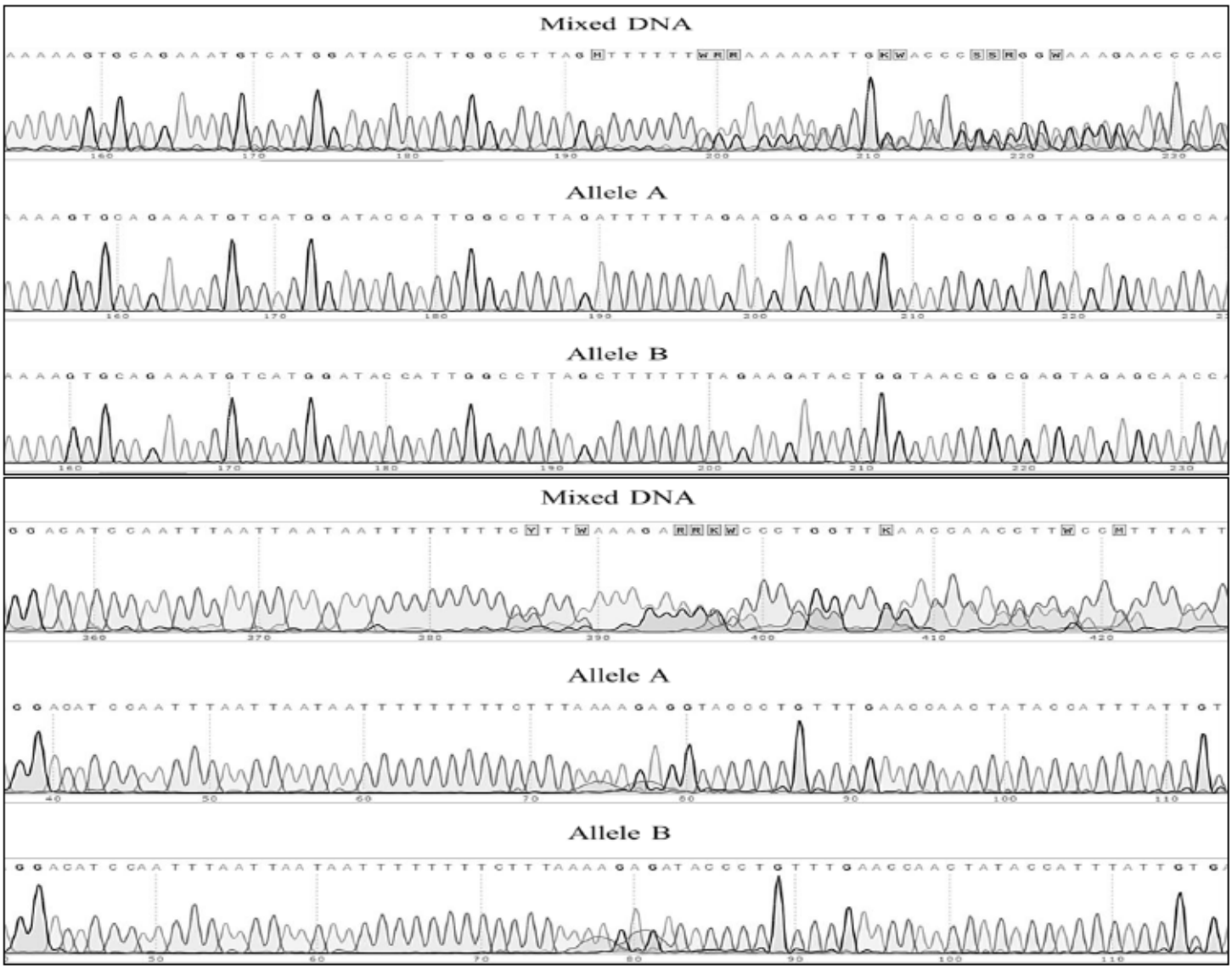

Figure 5 Electropherograms showing the GAP region resolved. The figure shows clearly the resolution of the two different sequences. (A) Forward sequences 5'>3': There is an indel in the T homopolymer, thus the mixed DNA had the two lectures overlapped. (B) Reverse sequences 3'>5': Results were similar to the Forward sequences, showing an indel in the homopolymer.

Table 1 Comparison of analyzed alleles with those of *S. cerevisiae* strains.

Strain	Allele A (%)	Allele B (%)
S. cerevisiae S288c	98.51	97.76
S. cerevisiae EC1118	99.14	99.37
S. cerevisiae YJM248	99.08	99.43
S. cerevisiae YJM270	98.97	99.43
S. cerevisiae YJM1078	99.08	99.43
S. cerevisiae YJM1252	99.08	99.43
S. cerevisiae YJM1450	99.08	99.31
S. cerevisiae YJM1463	98.97	99.54

CONCLUSION

In conclusion, our research shows that using a classical method, such as molecular cloning, problems of complex genomic sequencing projects can be solved in a simple way.

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BIOCONVERSION OF WATER-HYACINTH TO NUTRITIONALLY ENRICHED ANIMAL FEED BY SOLID STATE FERMENTATION USING *Pleurotus sajor-caju*

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ABSTRACT

This study was undertaken to improve nutritional values and digestibility of water-hyacinth by solid-state fermentation with a white rot fungi, *Pleurotus sajor-caju*. At the end of 56 days fermentation of $CaCO_3$ treated water-hyacinth, significant ($p<0.05$) changes of crude protein, lipid, carbohydrate, ash, lignin, cellulose, hemicellulose, cellulose-lignin ratio and reducing sugar contents were detected. Crude protein, ash, cellulose-lignin ratio and reducing sugar contents were increased by 685, 47, 106 and 680%, respectively. In contrary, crude fiber, lipid, carbohydrate, lignin, cellulose and hemicelluloses contents were decreased by 36.8, 72, 19, 72.33, 37.5 and 4.57%, respectively. Ascorbic acid and carotenoid were increased by 42.9 and 122.8%, respectively. At 49 days of fermentation, the crude water-hyacinth extract showed very high CMCase, avicelase and amylase, moderate cellobiase and very poor pectinase and xylanase activities. *In-vitro* dry matter digestibility was also increased by 76%. The study concluded with the finding that *P. sajor-caju* has the potential for efficient degradation of water-hyacinth to convert the lignocellulosic wastes into nutritionally improved animal feed.

Keywords: Bioconversion, water-hyacinth, solid-state fermentation, *Pleurotus sajor-caju*, animal feed

INTRODUCTION

Lignocellulosic wastes (LCW), refer to plant biomass wastes, are composed of cellulose, hemicellulose, and lignin. They may be grouped into different categories such as wood residues (including sawdust and paper mill discards), grasses, waste paper, agricultural residues (including straw, stover, peelings, cobs, stalks, nutshells, non food seeds, bagasse), domestic wastes (lignocelluloses garbage and sewage), food industry residues and municipal solid wastes (**Qi *et al.*, 2005; Roig *et al.*, 2006; Rodriguez *et al.*, 2008**). Even though LCW are considered as the largest reservoir of potentially fermentable carbohydrates on earth (Mtui and Nakamura, 2005) these are mostly wasted in the form of pre-harvest and post-harvest agricultural losses and wastes of food processing industries. Due to their abundance and renewability, there has been a great deal of interest in utilizing LCW for the production of protein rich food, fuel and other value-added products (**Pandey *et al.*, 2000; Mukherjee *et al.*, 2004; Foyle *et al.*, 2007**). The barrier to the production of valuable materials from LCW is the structure of lignocelluloses which has evolved to resist degradation due to crosslinking between the polysaccharides (cellulose and hemicellulose) and the lignin via ester and ether linkages (**Yan and Shuya, 2006; Xiao *et al.*, 2007**). Cellulose, hemicelluloses and lignin form a structure called microfibril, which are then organized into macrofibrils and gives structural stability in the plant cell (**Rubin, 2008**). The main target of lignocelluloses degradation, therefore, is to amend or eliminate structural and compositional hurdles for hydrolysis and subsequent degradation processes in order to improve digestibility, rate of enzymatic hydrolysis and product yields (**Mosier *et al.*, 2005; Hendriks and Zeeman, 2009**). The degradation can be achieved by single or combined implementation of mechanical, physico-chemical or biological treatments.

Microbial conversion of lignocelloses to energy and nutritionally enriched ruminant feed is becoming popular day-by-day. Water-hyacinth, a very fast-growing ubiquitous aquatic herb which is mainly used as cheap animal feed in Bangladesh has a promising possibility to convert as nutritionally improved animal feed after proper delignification and solid-state fermentation. The agro-waste grows so abundantly in rivers and other navigable waters where it obstructs the passage of boats and ships, and it is also troublesome in irrigation ditches. Its abundant growth sometimes threatens fish and other water life in the rivers and lakes by depriving them of oxygen and causing significant changes in aquatic habitats. The bioconversion of water-hyacinth is thus has a dual advantage of handling the waste for cleaner environment and production of value added animal feeds. White rot fungi, capable of degrading lignin, cellulose and hemicelluloses, have already been reported for efficient bioconversion of many lignocellosic wastes (**Anwar *et al.*, 2015; Dashtban *et al.*, 2009; Shrivastava *et al.*, 2014**). Conversion of water-hyacinth to ruminant feed by several white rot fungi including *Pleurotus. sajor-caju* has also been reported (**Mukherjee *et al.*, 2004; Mukherjee and Nandi, 2004**). However, combination of chemical and biological treatment is expected to further improve the bioconversion. In the present study, $CaCO_3$-pretreated water-hyacinth was used for SSF by *P. sajor caju* to enhance delignification and *in-vitro* dry matter digestibility in addition to several nutritional parameters. We further checked the augmentation of antioxidantive properties and enzyme activities of crude water-hyacinth extracts during the SSF.

MATERIALS AND METHODS

Preparation of substrates

Water-hyacinth collected from different sources were first cleaned off all dirt and unwanted materials. Then they were sun dried and cut into tiny pieces between 2-3 cm. It was stored at 5°C until used.

Pretreatment of substrates

500 g of untreated water-hyacinth was soaked with a calcium carbonate solution (2.67 g $CaCO_3$/L DH_2O). The substrates were left in soaking condition overnight. Then the lime solution was drained out by tap water. Treated substrates were then spread over aluminum foils and allowed to dry overnight at 60°C.

Collection and storage of *P. sajur-caju*

Stock culture of *Pleurotus sajur-caju* was obtained as Potato dextrose agar (PDA) slant from Microbiology and Industrial Irradiation Division of Bangladesh Atomic Energy Commission. The culture was maintained on PDA medium at 4°C and sub-cultured every 15 days.

Solid-state fermentation

P. sajor-caju was sub cultured from stock PDA slant to PDA plate. After 14 days of incubation at 30°C three pieces of mycelia growth (about 1 cm in diameter) were inoculated in 100 ml Erlenmeyer flask containing 50 ml PDA broth. The flask was incubated at 30°C in shaking condition in an orbital shaker for 7 days and then the inoculums was transferred in pre-sterilized soaked substrates (into 1000 ml Erlenmeyer flask) containing 20 g substrates and 50 ml distilled water and incubated at 30°C for 56 days.

Biochemical analyses

Water-hyacinth with different periods of fermentation were collected aseptically, oven dried at 60°C and used for biochemical analysis. The substrate without $CaCO_3$ treatment and SSF was used as control and also dried overnight at 60°C before biochemical analyses. Ash, fat, crude fiber and moisture contents were determined following the methods of **A.O.A.C (1980)**, while the crude protein contents (N×6.25) were determined using micro-kjeldahl method **(ISO 20483 2006)**. The carbohydrate contents were determined by Dubois et al. (1956). Gravimetric determination of lignin, cellulose and hemicellulose of the substrates were estimated according to **Sun *et al.* (1996)** and **Adsul *et al.* (2005)**. The cellulose to lignin ratio was also determined. Reducing sugar contents in control and fermented substrates at their various stages of fermentation were determined by the dinitrosalicylic acid (DNS) method **(Miller, 1959)**.

Determination of enzyme activity

The crude enzyme solution was obtained by soaking moldy substrate with considerable volume of 0.01 M acetate buffer (pH 5.5). The mixture was shaken for 2 h and centrifuged at 5000 rpm for 10.0 m to remove cells and residual substrate. The clarified extract representing crude enzyme was used for assaying endoglucanase (CMCase), exoglucanase (Avicilase), xylanase, **(Saddler *et al.*, 1987)** pectinase **(Shimizu and Kunoh, 2000)**, cellobiase **(Lowe *et al.*, 1987)** and Amylase **(Pandey *et al.*, 2000)** activities. Enzyme assays were carried out in triplicate using three culture replicates at 25°C. The enzymatic activities are expressed as international units (IU), defined as the amount of enzyme required producing 1 µmol product/minute, and are reported as IU/g substrate used in the SSF as described by **Shrivastava *et al.* (2011)**.

Quantification of antioxidants

Amount of ascorbic acid was quantified by spectrophotometric method after extraction with 3% HPO_3 as described in the **Methods of Vitamin Assay (1966)**. Total carotenoid was extracted in 80% acetone and absorption was taken at 663, 645 and 480 nm. Finally the amount of carotenoid was calculated using the following formula as described by **Hiscox and Isrealstam (1979)**.

$$\text{Total carotenoid (mg /g)} = A_{480} + (0.114 \times A_{663}) - (0.638 \times A_{645}) \times V / 1000 \times W$$

***In-vitro* dry matter digestibility (IVDMD)**

Dry matter digestibility was assessed following the methods of Tilley and Terry (1963) and Minson and McLeod (1972) and expressed as loss of dry matter. Ruminal fluid was obtained from a lactating goat after 4 h feeding on a mixed ration consisting of 75% grass forage and 25% grain mixture (20% ground corn, 4% soybean meal, 1% vitamin and mineral mix).

Statistical Analysis

Data from different biochemical analyses of non-fermented and fermented samples at different periods were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. Analyses were performed using statistical applications and differences and were considered significant at an alpha level of 0.05.The statistical program used was Stat-View® 5.0 (Mind Vision Software, Abaccus, Concepts, Inc. Berkeley, CA, USA).

RESULTS AND DISCUSSION

Changes in the proximal composition during SSF

The proximal composition of water-hyacinth was changed significantly after solid-state fermentation ($p<0.05$) compared to non-fermented one (Table 1). The crude fiber content decreased 36.86% after 56 days fermentation. This indicates secretion of cellulose/hemicellulose-degrading enzymes by the fungus during fermentation **(Lateef *et al.*, 2008)**. The protein content of fermented water-hyacinth was increased by 685.34% refereeing enormous increase of the fermenting fungal growth on water-hyacinth (Figure 1). The finding was in accordance with several previous reports **(Murata *et al.*, 1967; Hammond and Wood, 1985; Matsuo, 1997; Iluyemi et al., 2006; Moore *et al.*, 2007)**. Besides fungal growth, secretion of certain extracellular enzymes also contributed to the increase of protein **(Kadiri, 1999)**. Earlier studies of fungal growth on cassava byproducts, wheat straw, coffee husk, corn bran, and rice bran have also reported similar increase in protein content **(Leifa and Soccol *et al.*, 2001; Iyayi and Aderolu, 2004., Das and Mukherjee, 2007)**. The ash content was also found to increase with fermentation time and a total of 47.35% increased at the end. Since the ash content determination is a measure of mineral levels in the substrates, it can be inferred that SSF contributed to the elevation of mineral levels in the fermented products. Similar improvement of ash content, following fermentation of lignocelluloses has been reported by **Sanni and Ogbonna (1991)**, **Bressani (1993)**, and **O'Toole (1999)**. In contrary, **Fadahunsi *et al.* (2010)** and **Akinyele *et al.* (2011)** reported decrease of ash content due to SSF of agricultural wastes. Generally, fermentation led to reduction in the crude fat content. Here, the reduction was 72.65 % after 56 days SSF. In a similar study, the

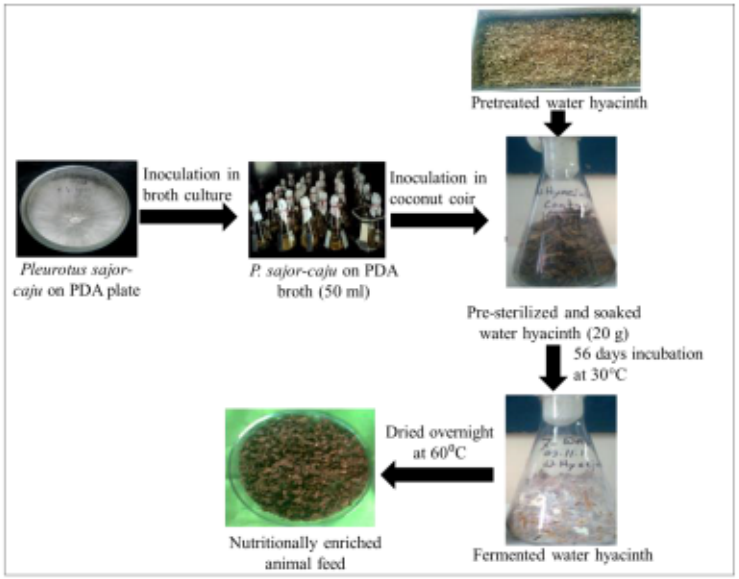

Figure 1 Biological conversion of water-hyacinth to nutritionally enriched animal feed. *P. sajor-caju* was sub cultured from PDA plate to 50 ml PDA broth and incubated at 30°C in shaking condition for 7 days. The inoculum was then transferred in pre-sterilized soaked substrates containing 20 g substrates and 50 ml distilled water and incubated at 30°C for 56 days. Final product was achieved after drying at 60°C for overnight.

Fat content of okara was reduced from 15 to 9% by fermentation with *N. intermedia* **(Matsuo, 1997)**. Previous studies have shown reduction in the lipid content of different substrates fermented with different microorganisms. During SSF, lipolytic strains assimilate lipid from substrates for biomass production and cellular activities leading to a general reduction of the overall lipid content **(Das and Weeks, 1979; Ejiofor and Okafor, 1987; Sanni and Ogbonna, 1991; Iluyemi *et al.*, 2006; Lateef *et al.*, 2008)**. The carbohydrate content of water-hyacinth was also decreased significantly because of the SSF. Carbohydrates are used through different biochemical processes by microorganisms to produce simple sugars during bioconversion of lignocelluloses **(Howard et al., 2003; Akinyele *et al.*, 2011)**.

Table 1 Proximate composition (% of dry substrate) of water-hyacinth at various period of solid-state fermentation with *P. sajor- caju*

Period of Incubation	Crude fiber	Protein	Ash	Lipid	Carbohydrates
control	4.07±0.16^{g}	2.32±0.19^{a}	9.80±0.28^{a}	1.06±0.16^{g}	76.80±0.60^{h}
7	3.89±0.04^{g}	5.68±0.25^{b}	10.16±0.23^{b}	0.905±0.02^{f}	75.17±0.36^{g}
14	3.70±0.04^{f}	8.89±0.33^{c}	10.68±0.02^{c}	0.867±0.01^{f}	71.52±0.18^{f}
21	3.57±0.06^{ef}	9.98±0.27^{d}	11.22±0.13^{d}	0.716±0.01^{e}	70.81±0.12^{f}
28	3.39±0.02^{de}	11.72±0.38^{e}	11.75±0.09^{e}	0.646±0.01^{de}	69.42±0.92^{e}
35	3.24±0.06^{d}	12.92±0.26^{f}	12.57±0.04^{f}	0.526±0.02^{cd}	68.3±0.34^{d}
42	3.04±0.08^{c}	14.42±0.27^{g}	12.67±0.02^{f}	0.444±0.02^{bc}	67.2±0.27^{c}
49	2.78±0.06^{b}	16.09±0.49^{h}	13.86±0.05^{g}	0.353±0.01^{ab}	64.45±0.13^{b}
56	2.57±0.06^{a}	18.22±0.93^{i}	14.44±0.08^{h}	0.290±0.01^{a}	62.01±0.74^{a}

Results are expressed as mean ± SD (standard deviation) of three independent experiments. Values in the same column with different superscripts are significantly different at $p< 0.05$.

The reducing sugar content of water-hyacinth was increased dramatically and correlated directly with increase of biomass and decrease of carbohydrates during 56 days fermentation period (Table 2). The reducing sugar content of fresh water-hyacinth was found to increase up to 49 days of fermentation indicating enzymatic degradation of cellulose, hemicelluloses and pectin fractions of the substrate (**Sherief *et al.*, 2010**). However, the decreased free sugar content after 49 days fermentation can be explained by decreased rate of the degradation as compared to the rate of free sugar metabolism by *P. sajor caju*. This submission corroborates the findings of **Sanni and Ogbonna (1991)** where they reported a sharp decrease of enzymatic activity at 24h of fermentation during the production of 'Owoh" from cotton seed.

Table 2 Lignin, cellulose, hemicelluloses, C/L and reducing sugar contents (% of dry substrate) of water-hyacinth at different period of SSF by *P. sajor caju*.

Period of Incubation (days)	Lignin	Hemicelluloses	Celluloses	Cellulose and Lignin ratio (C/L)	Reducing sugar
control	15.25±0.85^{e}	18.15±1.15^{e}	23.75±1.52^{e}	1.56±0.22^{a}	0.30±0.02^{a}
7	12.77±0.80^{d}	17.32±0.93^{de}	20.32±0.92^{d}	1.60±0.176^{ab}	0.68±0.03^{b}
14	10.23±0.63^{c}	16.89±0.80$_{cde}$	19.58±1.76^{cd}	1.91±0.057^{bc}	0.81±0.03^{c}
21	9.84±0.70^{c}	16.78±1.03^{cde}	18.02±0.49^{bcd}	1.84±0.051^{abc}	1.03±0.02^{d}
28	9.70±0.42^{c}	15.67±0.95^{abcd}	17.67±0.95^{bc}	1.82±0.078^{abc}	1.07±0.03^{d}
35	9.19±0.68^{c}	16.03±0.50^{bcde}	17.06±0.56^{ab}	1.87±0.014^{bc}	1.51±0.01^{e}
42	7.67±0.47^{b}	14.86±0.76^{abc}	16.03±0.51^{ab}	2.08±0.078^{cd}	1.89±0.08^{f}
49	6.75±0.35^{b}	13.95±0.88^{ab}	15.02±0.49^{a}	2.22±0.078^{d}	2.88±0.11^{h}
56	4.22±0.16^{a}	13.75±1.06^{a}	14.83±0.70^{a}	3.52±0.042^{e}	2.34±0.04^{g}

Results are expressed as mean ± SD (standard deviation) of three independent experiments. Values in the same column with different superscripts are significantly different at $p< 0.05$.

Degradation of lignin, cellulose and hemicelluloses

The chemical pretreatment of water-hyacinth with $CaCO_3$ prior to SSF enhanced the delignification and resulted in a decrease of lignin content from 15.25% of total dry weight to 14% (8.2% loss). While comparing the contents of lignin, hemicelluloses and cellulose during SSF, a significant degrease ($p< 0.05$) of all these contents were observed. However, cellulose and lignin ratio (C/L ratio) of fermented agro-industrial wastes was significantly increased ($p< 0.05$) compared with their unfermented samples. The percentage of lignin content was decreased by 72.33 % (Table 2) for SSF indicating the ability of *P. sajor-caju* to bulk of ligninases production such as laccases and peroxidases (**Leonowicz *et al.*, 1999; Baldrian *et al.*, 2005; Hoegger *et al.*, 2007**) while fermenting water-hyacinth. The finding was in accordance with the previous reports of **Lechner and Papinutti (2006)** and **Sherief *et al.* (2010)** where lignolytic activities of fermenting microorganisms were found during biodegradation of rice straw, saw dust, wheat straw, coffee pulp and banana leaves. The percentage of cellulose was found to reach 14.83% of the total dry weight at the end of 56 days fermentation (Table 2) after a reduction of 37.5% from the initial cellulose content that indicates the increased production of cellulases. Cellulose degradation is a usual phenomenon during SSF of lignocelluloses as reported by **Bisaria *et al.* (1997)**, **Sherief *et al.* (2010)** and **Jahromi *et al.* (2011)**. Unlike cellulose, hemicellulose degradation was found lower and at the end the reduction was 24.24% compared to non-fermented one. The decrease in the values of hemicellulose could be indicative of the degradation of the cell wall component of the substrates produced by the extracellular enzymes (xylanase, xylosidase, arabinase and pectinase) of the fungi used.

Cellulolytic enzyme activities

Edible mushrooms (*P sajor-caju* and *P. pulmunarium*) are able to convert a wide variety of lignocellulose materials due to the secretion of extracellular enzymes (**Buswell *et al.*, 1996 and Rajarathnan *et al.*, 1998**). Increase of free sugar and decrease of cellulose and hemicellulos (Table 2) during SSF indicated the presence of degradation cellulolytic enzyme activities of *P. sajor-caju* while growing on water-hyacinth. Therefore, crude enzymatic activities of *P. sajor-caju* were measured at the period of 49 days SSF as maximum reducing sugar was found at this point. CMCase, avicelase and cellobiase activities were 1.23, 0.92 and 0.31 IU/g respectively (Figure 2). These activities directly correlate with the degradation of cellulose. A very low enzymatic activity of xylanase was expected as the hemicelluloses degradation was lower compared to cellulose degradation. However, the fungus also showed low pectinase activity and moderate amylase activity. Very low xylanase activity was also reported by **Kumar *et al.*, (1997)** during SSF of Sago hampus, a starchy lignocellolosic by-product prepared from sago palm.

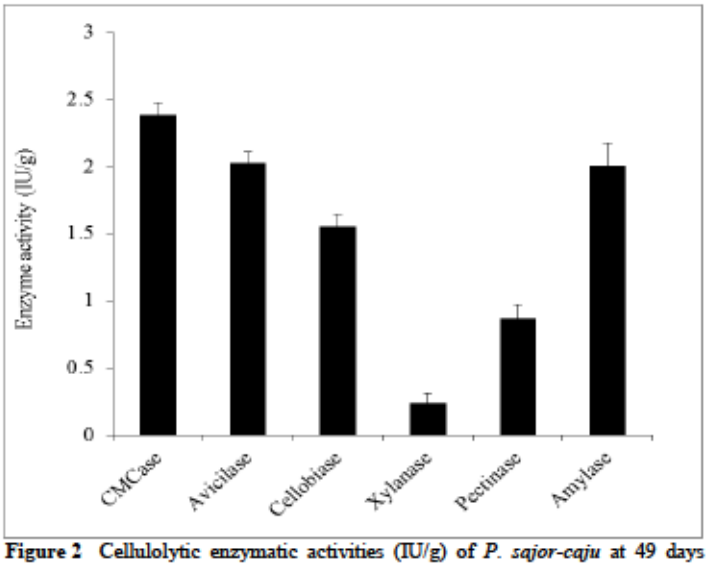

Figure 2 Cellulolytic enzymatic activities (IU/g) of *P. sajor-caju* at 49 days SSF with water-hyacinth. Values are mean±SD of three independent experiments.

Improvement of antioxidative nature and *in-vitro* digestibility

We also checked the change of dry matter, anti-oxidative properties and *in-vitro* dry matter digestibility (IVDMD) of water-hyacinth as a consequence of SSF (Table 3). Increase of dry matter by 9.3% was because of increased biomass as a mycelial growth of the fungus. Ascorbic acid was improved by 42%. Growth of *P. sajor-caju* also contributed to improving significant level of total carotenoid by 122.8%. More importantly, the IVDMD was changed in significant level. Improved IVDMD of water hyacinth after solid-state fermentation has also been reported by **Mukherjee *et al.* (2004)**, however, in our study the improvement was higher as we used a chemical pretreatment which increased the delignification. This result was supported by the findings that digestibility is usually inversely related to the lignin concentration (**Kamra and Zadrazil,**

1985). Karunanandaa *et al.* (1995) also reported higher digestibility of paddy straw because of faster delignification than other lignocellulosic wastes by mutant strains of *P. forida* in SSF. As ruminal microbes do not secrete any ligninolytic enzyme (Zadrazil *et al.*, 1995), the chemical pretreatment aided in lignin reduction which facilitated the degradation of structural carbohydrates of water hyacinth by solid state fermentation. Thus, the SSF used in this study helped to accumulate higher amount of soluble sugars through bioconversion which will be easily digestible by ruminants.

Table 3 Amounts of total dry wt, ascorbic acid, total carotenoid and *in-vitro* digestibility in water hyacinth before and after SSF

Type of sample	Total dry wt (g)	Ascorbic acid (mg/g)	Total Carotinoid (mg/g)	*In-vitro* digestibility (% of dry substrate)
control	20.00±0.57[a]	0.0573±0.006[a]	0.105±0.01[a]	20.25 ±0.45[a]
Fermented substrates	21.86±0.83[b]	0.0819±0.018[a]	0.234±0.026[b]	35.65±0.75[b]

Results are expressed as mean ± SD (standard deviation) of three independent experiments. Values in the same column with different superscripts are significantly different at $p < 0.05$.

CONCLUSION

The present study revealed that solid state fermentation of pre-treated water-hyacinth not only improved nutritive values such as protein and available polysaccharide fractions as energy source for ruminants but also made it more digestible due to higher delignification. In addition, the fermented product was also rich in some anti-oxidative agents. Therefore the bioconverted product can be used as nutritionally improved animal feed after an *in-vivo* feeding trial and toxicity tests.

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GENETIC DIVERSITY ANALYSIS OF MAIZE (*ZEA MAYS* L.) USING SCoT MARKERS

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ABSTRACT

Molecular characterization is frequently used by maize breeders as an alternative method for selecting more promising genotypes and reducing the cost and time needed to develop hybrid combinations. In the present investigation 20 genotypes of maize from Czechoslovakia, Hungary, Poland, Union of Soviet Socialist Republics, Slovakia and Yugoslavia were analysed using 5 Start codon targeted (SCoT) markers. These primers produced total 29 fragments across 20 maize genotypes, of which 22 (77.90 %) were polymorphic with an average of 4.40 polymorphic fragments per primer and number of amplified fragments ranged from 4 (SCoT 8) to 7 (SCoT 12 and SCoT 23). The polymorphic information content (PIC) value ranged from 0.652 (ScoT 8) to 0.816 (SCoT 23) with an average of 0.738. The dendrogram of 20 maize genotypes based on SCoT markers using UGMA algorithm was constructed. The hierarchical cluster analysis divided maize genotypes into two main clusters. Unique 2 maize genotype Slovenska žltá and Slovenska krajová velkozrná, originated from Slovak Republic, separated from others. Cluster 2 containing 18 genotypes was divided into two main subclusters. Subcluster 2a contained two Poland genotypes Przebedowska Burskynowa and Zloty Zar, two genotypes of Union of Soviet Socialist Republics- Partizanka and Krasnodarskaja and one Czechoslovakian genotypes Milada. In subcluster 2b were grouped 13 maize genotypes. The present study shows effectiveness of employing SCoT markers in analysis of maize, and would be useful for further studies in population genetics, conservation genetics and genotypes improvement.

Keywords: Dendrogram; Maize; Molecular markers; SCoT analysis; PIC

INTRODUCTION

Maize (*Zea mays* L.) is one of the world's most important crop plants following wheat and rice, which provides staple food to large number of human population in the world (**Ahmad *et al.*, 2011; Iqbal, *et al.*, 2015**). Determining genetic diversity can be based on agronomic, morphological, biochemical, and molecular types of information, among others (**Goncalves *et al.*, 2009**). Molecular characterization is frequently used by maize breeders as an alternative method for selecting more promising genotypes and reducing the cost and time needed to develop hybrid combinations (**Garcia *et al.*, 2004**). In recent years, a number of molecular markers have been employed for genetic diversity evaluation, genetic mapping, and quantitative trait locus analysis. These types of molecular techniques included random amplified polymorphic dna (RAPD) (**Petrovičová *et al.*, 2015; Štefúnová *et al.*, 2015; Vivodík *et al.*, 2015; Žiarovská *et al.*, 2016**), amplified fragment length polymorphism (AFLP) (**Molin *et al.*, 2013**), inter-simple sequence repeat (ISSR) (**Idris *et al.*, 2012; Žiarovská *et al.*, 2013**) and simple sequence repeats (SSR) (**Shehata *et al.*, 2009; Lanciková *et al.*, 2015; Balážová *et al.*, 2016; Vivodík *et al.*, 2016**).

Recently, a simple novel DNA marker technique namely start codon targeted (SCoT) polymorphism, was developed by **Collard and Mackill (2009)**. Primers for SCoT marker analysis were designed from the conserved region surrounding the translation initiation codon, ATG (**Joshi *et al.*, 1997; Sawant *et al.*, 1999**). Single 18-mer oligonucleotides were used as both forward and reverse primer for PCR, and the annealing temperature was set at 50 °C. The amplicons were resolved using standard agarose gel electrophoresis. Suitability of SCoT markers for the construction of genetic maps, fingerprinting and phylogenetic studies has been proved by many authors in many crops, such as tomato (**Shahlaei *et al.*, 2014**), citrus (**Mahjbi *et al.*, 2015**), date palm (**Al-qurainy *et al.*, 2015**), castor (**Kallamadi *et al.*, 2015**) and mango (**Gajera *et al.*, 2014**).

The goals of this study were to examine the effectiveness of SCoT markers for analysis of genetic diversity of maize and to study genetic relationships among 20 maize accessions originating from various geographic regions of Europe.

MATERIAL AND METHODS

Plant material

Twenty genotypes of old maize lines originating from six different geographical areas (Table 1) (CZE - Czechoslovakia, HUN - Hungary, POL - Poland, SUN – Union of Soviet Socialist Republics, SK – Slovakia, YUG - Yugoslavia) of Europe were obtained from the Gene Bank Praha-Ruzyně (Czech Republic) and from the Gene Bank in Piešťany (Slovakia). Maize genotypes were grown in a growth chamber on humus soil. Genomic DNA was isolated from the 14 days leaves with GeneJET Plant Genomic DNA Purification Mini Kit.

SCoT amplification

A total of 5 SCoT primers developed by **Collard and Mackill (2009)** were selected for the present study (Table 2). Each 15-µL amplification reaction consisted of 1.5 µL (100 ng) template DNA, 7.5 µL Master Mix (Genei, Bangalore, India), 1.5 µL 10 pmol primer, and 4.5 µL distilled water. Amplification was performed in a programmed thermocycler (Biometra, Germany) using the following program: 94°C for 3 min; 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; a final extension at 72°C for 5 min. Amplified products were separated in 1.5% agarose in 1× TBE buffer. The gels were stained with ethidium bromide and documented using gel documentation system UVP PhotoDoc-t® camera system. A dendrogram was constructed based on hierarchical cluster analysis using the unweighted pair group method with arithmetic average (UPGMA). For the assessment of the polymorphism between genotypes maize and usability SCoT markers in their differentiation we used polymorphic information content (PIC) (**Weber, 1990**).

Table 1 List of 20 analyzed genotypes of maize

Genotypes	Country of origin	Year of registration
1. Feheres Sarga Filleres	Hungary	1965
2. Mindszentpusztai Feher	Hungary	1964
3. Zakarpatskaja	Union of Soviet Socialist Republics	1964
4. Przebedowska Burskynowa	Poland	1964
5. Krasnodarskaja	Union of Soviet Socialist Republics	1964
6. Mesterhazy Sarga Simaszemu	Hungary	1964
7. Slovenska biela perlova	Czechoslovakia	1964
8. Zuta Brzica	Yugoslavia	1975
9. Zloty Zar	Poland	1964
10. Slovenska Florentinka	Czechoslovakia	1964
11. Juhoslavska	Yugoslavia	1964
12. Kostycevskaja	Union of Soviet Socialist Republics	1964
13. Mindszentpusztai Sarga Lofogu	Hungary	1964
14. Stodnova	Czechoslovakia	1964
15. Slovenska žltá	Slovak Republic	1964
16. Slovenska krajová velkozrná	Slovak Republic	1964
17. Partizanka	Union of Soviet Socialist Republics	1964
18. Voroneskaja	Union of Soviet Socialist Republics	1964
19. Kocovska Skora	Slovak Republic	1964
20. Milada	Czechoslovakia	1964

RESULTS AND DISCUSSION

In this work, all 5 SCoT primers used for analysis of 20 European old maize genotypes produced amplification products and all resulted in polymorphic fingerprint patterns. Five primers produced 29 DNA fragments (Figure 1) with an average of 5.80 bands per primer (Table 2). Out of the total of 29 amplified fragments, 22 (77.90 %) were polymorphic, with an average of 4.40 polymorphic bands per primer. From these five primers, primers SCoT 12 and SCoT 23, respectively, were the most polymorphic, where 7 polymorphic amplification products were detected. The lowest number of amplified polymorphic fragments (4) was detected by primer SCoT 8. To determine the level of polymorphism in the analysed group of maize genotypes, polymorphic information content (PIC) was calculated (Table 2). The polymorphic information content (PIC) value ranged from 0.652 (SCoT 8) to 0.816 (SCoT 23) with an average of 0.738. The dendrogram of 20 maize genotypes based on SCoT markers using UGMA algorithm was constructed (Figure 2). The hierarchical cluster analysis divided maize genotypes into two main clusters. Unique 2 maize genotype Slovenska žltá and Slovenska krajová velkozrná, originated from Slovak Republic (cluster 1), separated from others. Cluster 2 containing 18 genotypes was divided into two main subclusters (2a and 2b). Subcluster 2a contained two Poland genotypes Przebedowska Burskynowa and Zloty Zar, two genotypes of Union of Soviet Socialist Republics- Partizanka and Krasnodarskaja and one Czechoslovakian genotypes Milada. In subcluster 2b were grouped 4 genotypes from Hungary (30.77%), 3 genotypes from Czechoslovakia (23.08%), 3 genotypes from Union of Soviet Socialist Republics (23.08%), 2 genotypes from Yugoslavia (15.38%) and 1 genotypes from Slovak Republic (7.70%).

Table 2 Statistical characteristics of the SCoT markers used in maize

SCoT Primers	Primer sequence (5´-3´)	TNoB	NoPB	PoPB	PIC
SCoT 6	CAACAATGGCTACCACGC	5	4	80.00	0.729
SCoT 8	CAACAATGGCTACCACGT	4	4	100.00	0.652
SCoT 9	CAACAATGGCTACCAGCA	6	4	66.66	0.780
SCoT 12	ACGACATGGCGACCAACG	7	5	71.43	0.715
SCoT 23	CACCATGGCTACCACCAG	7	5	71.43	0.816
Average		**5.80**	**4.40**	**77.90**	**0.738**
Total		**29**	**22**	-	-

TNoB-Total number of bands, NoPB- Number of polymorphic bands, PoPB- Percentage of polymorphic bands (%), PIC- Polymorphic information content

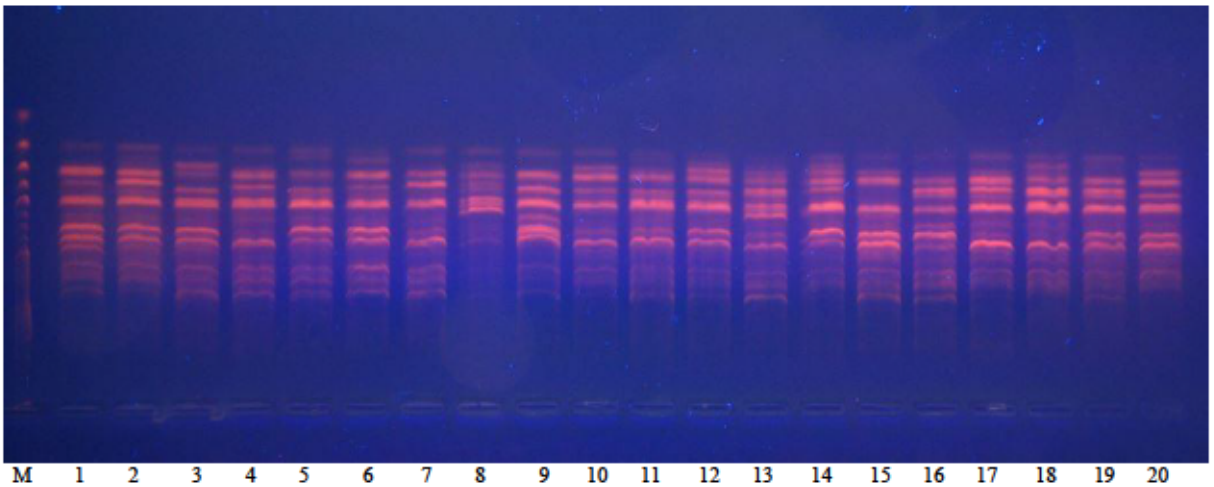

Figure 1 PCR amplification products of 20 genotypes of maize produced with SCoT marker SCoT 12. Lanes 1 - 20 are maize genotypes (Table 1) and M- 100 bp DNA ladder

Level of polymorphism in analysed maize genotypes was determined by calculated polymorphic information content (PIC) (Table 2). The PIC values ranged from 0.374 (SCoT 45) to 0.846 (SCoT 28) with an average of 0.739. Similar values of PIC were detected by other authors (**Luo *et al.*, 2012; Arya *et al.*, 2014; Gajera *et al.*, 2014; Que *et al.*, 2014; Gao *et al.*, 2014; Fang-Yong *et al.*, 2014; Jiang *et al.*, 2014; Huang *et al.*, 2014; Satya *et al.*, 2015**) and these values presented a high level of polymorphism of genotypes detected by SCoT markers. **Huang *et al.*, (2014)** assessed the genetic diversity of six Hemarthria cultivars using seven SCoT primers, which together amplified 105 bands with an average of 15 bands per sample. Start codon-targeted markers were utilized by **Gajera *et al.*, (2014)** who used 19 SCoT markers for characterization and genetic comparison among 20 mango cultivars. These primers produced total 117 loci across 20 cultivars, of which 96 (79.57 %) were polymorphic. In the study **Que *et al.*, (2014)**, used 20 start codon targeted (SCoT) marker primers to assess the genetic diversity among 107 sugarcane accessions within a local sugarcane germplasm collection. These primers amplified 176 DNA fragments, of which 163 were polymorphic (92.85%). The aim of **Gao *et al.*, (2014)** was to estimate the genetic diversity across 43 varieties of Lycoris. Of 57 SCoT primers screened, 23 SCoT primers were identified to be high polymorphism. **Fang-Yong *et al.*, (2014)** assessed the genetic diversity of 31 germplasm resources of Myrica rubra from Zhejiang Province, the major gathering site and the largest producer of M. rubra in China using start codon-targeted polymorphism (SCoT) markers. **Satya *et al.*, (2015)** used 24 start codon targeted (SCoT) markers to assess genetic diversity and population structure of indigenous, introduced and domesticated ramie (*Boehmeria nivea* L. *Gaudich.*). **Jiang *et al.*, (2014)** used start codon-targeted (SCoT) markers to analyze the diversity and genetic relationships among 95 orchardgrass accessions. In total, 273 polymorphic bands were detected with an average of 11.4 bands per primer. In the study **Zhang *et al.*, (2015)**, used SCoT markers to study the genetic diversity and relationships among 53 Elymus sibiricus accessions.

Studies of genetic diversity across individuals of plant have been realized by different PCR-based DNA marker methods: random amplified polymorphic DNA (RAPD) (**Molin *et al.*, 2013; Balážová *et al.*, 2016; Kuťka Hlozáková *et al.*, 2016**), simple sequence repeat (SSR) (**Terra *et al.*, 2011; Molin *et al.*, 2013; Gálová *et al.*, 2015; Balážová *et al.*, 2016**), amplified fragment length polymorphism (AFLP) (**Molin *et al.*, 2013**), inter-simple sequence repeat (ISSR) (**Žiarovská *et al.*, 2013; Molin *et al.*, 2013**). These methods are technically simple, fairly cheap and generate a relatively large number of markers per sample. **Molin *et al.*, (2013)** pointed that in general, a higher number of investigated accessions and more varied genetic background result in a higher expected polymorphic rate. Start codon targeted polymorphism (SCoT) is a simple and novel marker system first described by **Collard and Mackill (2009)**, which is based on the short conserved region flanking the ATG translation start codon in plant genes. The higher primer lengths and subsequently higher annealing temperatures ensure higher reproducibility of SCoT markers, compared to RAPD markers (**Rajesh *et al.*, 2015**). **Gorji *et al.*, (2011)** presented that SCoTs markers were more informative and effective, followed by ISSRs and AFLP marker system in in fingerprinting of potato varieties.

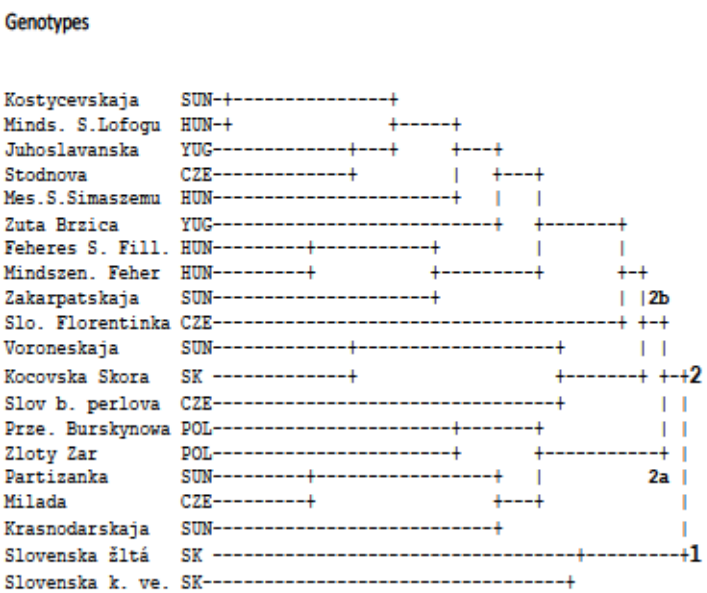

Figure 2 Dendrogram of 20 maize genotypes prepared based on 5 SCoT markers.

CONCLUSION

The present work is the first report on genetic variability of maize using SCoT markers. In summary, SCoT marker analysis was successfully developed to evaluate the genetic relationships among the genus maize accessions originated from various regions. The hierarchical cluster analysis showed that the maize genotypes were divided into 2 main clusters. Unique 2 maize genotype Slovenska žltá and Slovenska krajová velkozrná, originated from Slovak Republic (cluster 1), separated from others. Cluster 2 containing 18 genotypes was divided into two main subclusters (2a and 2b). Subcluster 2a contained two Poland genotypes Przebedowska Burskynowa and Zloty Zar, two genotypes of Union of Soviet Socialist Republics- Partizanka and Krasnodarskaja and one Czechoslovakian genotypes Milada. In subcluster 2b were grouped 13 maize genotypes. Polymorphism revealed by SCoT technique was so abundant and could be used for molecular genetics study of the maize accessions, providing high-valued information for the management of germplasm, improvement of the current breeding strategies, and conservation of the genetic resources of maize species.

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BIOCHEMICAL AND MICROBIOLOGICAL CHANGES THROUGHOUT THE RIPENING OF ARGENTINEAN FRESH GOAT'S MILK CHEESES MADE WITH NATIVE CULTURES

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ABSTRACT

Two different cultures with native lactic acid bacteria (LAB) were designed, S (starter culture consisting of *Lactobacillus (L.) rhamnosus* UNSE308, *L. delbrueckii* subsp. *bulgaricus* UNSE309, *Streptococcus (S). thermophilus* UNSE314, *S. thermophilus* UNSE321) and A (starter culture S plus adjunct culture consisting of *L. plantarum* UNSE316, *L. plantarum* UNSE317, *Pediococcus (P). pentosaceus* UNSE22, *P. pentosaceus* UNSE253). Cultures S, A and C (lyophilized commercial culture of *Streptococcus thermophillus*) were used to manufacture fresh goat cheeses (CS, CA and CC) from pasteurized milk. The impact of these cultures on the microbiological, biochemical and physicochemical parameters were evaluated during ripening (30 days). Results evidenced that CC, CA and CS did not show significant differences in protein and fat content, NaCl, acidity, fat acidity and aw, at the same time of ripening. All experimental cheeses underwent moderate lipolysis since moderate levels of free fatty acids (FFA) at the end of ripening were detected (~1.5 g kg^{-1}) and made an important contribution to characteristic flavour and aroma. In all cases, a slight proteolysis was detected; CC showed the lowest levels during ripening. Values of NaCl (1.27%) were in accordance with those reported in most cheeses. During ripening, mesophilic LAB counts in MRS agar increased nearly 1 log cycle, total coliforms per gram at 30°C showed counts below the maximum allowed by Argentinean legislation. Overall impression of CS and CA was qualified as good compared to CC that was scored as regular. The fresh goat cheeses made with native cultures presented suitable typical flavour and satisfying overall sensorial characteristics.

Keywords: Fresh goat cheese, native starter culture, native adjunct culture, physicochemical analysis, microbiological analysis

INTRODUCTION

The total of goats distributed around the world is 940 million animals, mainly distributed between Asia and Africa. In South America, there are 20.6 million goats, Brazil ranks first with 8.78 million goats and Argentina, second with 4.38 million goats (**FAO, 2013**). In 2012, milk production in Argentina has been evaluated in 2 million litres, mostly processed into cheeses in the northwest and central regions (**AACREA-Agroalimentos**). Goat's cheese production has increased greatly since the 1990s (**SAGPyA, 2007**).

Although some goat's milk cheeses are produced under artisanal conditions, most are manufactured at industrial scale (**AACREA-Agroalimentos**), especially semi-hard goat cheese, receiving little attention the fresh goat cheese. Although these cheeses have been produced in Argentina for more than a century ago, they have not been characterized as traditional cheeses. At present, little data on the biochemical changes produced during ripening which are responsible for the organoleptic and sensory characteristics of fresh goat cheese have been reported. According to Argentinean legislation, fresh cheeses must be made from pasteurized milk (**ANMAT, 2014**). But, pasteurization not only destroys pathogens but also the naturally occurring bacteria that contribute to flavor and might convey health benefits **Tunick, (2014)**. In this way, the drastic reduction in the number of propionibacteria and facultatively heterofermentative lactobacilli produced by milk pasteurization, modifies the catabolism of many organic acids, such as lactate and citrate (**Buffa *et al.* 2004**). Thus, the resulting cheese develops a less intense flavour and ripens more slowly than raw milk cheese (**Fox and Mc Sweeney, 2004**).

One way to intensify the flavour and improve the technological process is by the addition to pasteurized milk of adjunct and starter cultures, composed of selected lactic acid bacteria (LAB) (**Coppola *et al.*, 2008**). Starter cultures cause rapid acidification of the milk through the production of organic acids, mainly lactic acid (**Leroy and De Vuyst, 2004**).

The composition of most mesophilic starters include species of *Lactococcus* and *Leuconostoc*, thermophilic organisms of starter belong to two genera, *Lactobacillus* (*L.*) and *Streptococcus* (*S.*): *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *lactis*, *L. helveticus* and *L. plantarum* (**Mäyrä-Mäkinen and Bigret, 2004; Reinheimer and Zalazar, 2006**). Adjunct cultures are mostly composed of facultative heterofermentative mesophilic lactobacilli including species such as *L. casei, L. paracasei, L. rhamnosus* and *L. plantarum*, as well as pediococci, *Leuconostoc* and micrococci (**Burns *et al.* 2012**) grown during the ripening process (**Settanni and Moschetti, 2010**).

In previous works, we have studied biochemical, technological and healthy properties of LAB isolated from goat milk and artisanal cheeses from north-west Argentina **Taboada *et al.* (2014a, b, 2015)** in order to select appropriate starter and adjunct cultures.

The purpose of the present work was to employ native and commercial cultures in the manufacture of fresh goat cheeses made from pasteurized milk and to evaluate the impact of these cultures on the microbiological, biochemical and physicochemical parameters of the end product.

MATERIALS AND METHODS

Microorganisms

The native strains used in this study were provided by the Universidad Nacional de Santiago de Estero (UNSE), Argentina. They were isolated from Argentinean goat milk and artisanal goat cheeses, phenotypically identified and carefully selected by their technological properties **Taboada *et al.* (2014 a, b)**. The following strains were used: *L. rhamnosus* UNSE308, *L. delbrueckii* subsp. *bulgaricus* UNSE309, *S. thermophilus* UNSE314, *S. thermophilus* UNSE321, *L. plantarum* UNSE316, *L. plantarum* UNSE317, *P. pentosaceus* UNSE22, *P. pentosaceus* UNSE253.

Acidifying ability test

This test was performed to determine the acidifying capacity of strains in similar processing conditions to those in cheesemaking. **Hynes *et al.* (2000)** protocol was used with modifications. Bottles containing 100 mL of pasteurized goat milk (at 65°C for 30 min) were kept in a water bath at 38°C. Then, 0.2 g/L $CaCl_2$ (Merck, Darmstadt, Germany) was added, with a 2% (v/v) inoculum of an active culture with 10^8 cfu mL^{-1} of each of the strains mentioned above in 2.1. Then, 0.014 g/L chymosin (MAXIREN 150, Delft, The Netherlands) was added to coagulate milk. Coagulation time was controlled by rocking the bottles gently to test adhesion of casein to their sides. After coagulation, the bottles were maintained in a water bath at 38°C to attain the appropriate curd strength. Cell growth and proliferation were followed by means of pH measurements. The pH determinations were made every 30 min, from inoculation until attaining the appropriate curd strength, at a pH of about 5.2, according to **Janhøj and Qvist (2010)**.

Strain composition of native cultures

Based on the acidifying activity of individual strains and compatibility tests (**Collins *et al.* 1991**) different combinations of strains were used in cheesemaking, according to the following schedule:

Strain composition of native cultures

Strains (%, v/v)	Starter culture (S) (%, v/v)	Starter culture plus adjunct culture (A)
L. rhamnosus UNSE308	30	30
L.delbrueckii subsp. *bulgaricus* UNSE309	30	30
S. thermophilus UNSE314	20	18
S. thermophilus UNSE321	20	17
L. plantarum UNSE316	0	1.25
L. plantarum UNSE317	0	1.25
P. pentosaceus UNSE22	0	1.25
P. pentosaceus UNSE253	0	1.25

In relation to the starter culture, for the selection of the species the criteria of **Kosikowski, (1978)** were followed.
Adjunct cultures contribute to desired cheese ripening events through their metabolic activities such as proteolysis and lipolysis which influence flavour and texture development of the product (**Barouei *et al.* 2011**). In previous works (**Taboada *et al.* 2014a**), both esterase and esterase-lipase activity were detected in the strains selected as adjunct cultures. Since it is desired to obtain a product that is accepted by the majority of consumers, intense lipolysis would not be desirable. According to our experience, native culture A was designed as follows, composed of 95% (v/v) starters plus 5% (v/v) adjunct strains, meaning 1.9 x 10^6 cfu mL^{-1} of starter strains and 10^5 cfu mL^{-1} of adjunt strains, in vat.

Optimal inoculation proportion test

In order to obtain the optimal proportion to inoculate the native cultures A and S, the acidifying ability test was performed by using an inoculum of 1, 1.5 and 2% (v/v).

Milk for cheese manufacture

Goat's milk (Creole breed) provided by a local farm was refrigerated and transported at 4°C to the pilot plant of Instituto de Ciencia y Tecnología de Alimentos, UNSE (Santiago del Estero, Argentina). The chemical composition was expressed in percent (w/v) and determined by Lactostar analyser (Funke Gerber Lactostar, Funke-Dr. N. Gerber Labortechnik GmbH, Berlin, Germany). The official methods AOAC (Association of Official Analytical Chemists) were used for acidity (**Method 947.05, AOAC 2006**) and density (**Method 925.22, AOAC 2006**) determinations. The pH value was determined using a Metrohm 962 pHmeter (Herisau, Switzerland).

Cheese manufacturing protocol

Fresh cheeses were manufactured using native starter culture (S) or native starter plus adjunct culture (A) or a lyophilized commercial culture (C) of *S. thermophillus* (type DVS, Diagramma SA, Santa Fe, Argentina). The native strains and the lyophilized commercial culture were previously activated in MRS broth (Merck, Darmstadt, Germany) later they were multiplied in pasteurized goat milk with incubation at 35 °C until coagulation. Cultures with 10^8 cfu mL^{-1} were used for cheese manufacturing.
Fresh experimental cheeses were prepared using the following standard protocol: raw goat milk (35 L) was batch pasteurized at 65°C for 30 min and after cooling at 38°C, 0.2 g/L $CaCl_2$ (Merck, Darmstadt, Germany) was added, followed by a 2% (v/v) inoculum of S or A cultures, or a 1% (v/v) of C culture. Milk coagulation was achieved by adding 0.014 g/L chymosin (MAXIREN 150, Delft, The Netherlands). After 40 min of rest, the curd was cut to corn grain size and placed in cylindrical moulds (10 cm height, 12 cm diameter), pressed for 120 min. Cheeses were placed in cold storage at 5°C for 12 h. Then, cheeses were salted by immersion in 16% (w/v) NaCl sterile solution at 5°C for 2 h. Ripening was carried out at 12°C and 85% relative humidity for 30 days, being vacuum packaged at the end of ripening. Cheeses of approximately 700 g were obtained.
Three independent trials for each type of cheese (CS, cheese manufactured with native culture S; CA, cheese manufacture with native culture A; CC, cheese manufactured with commercial culture C) were made on different elaboration days, during a period of 3 months. Samples were taken at days 1 and 30 for physicochemical and microbiological analysis. Throughout the ripening period, the cheeses were turned over daily or every second day and, when necessary, the surface was brushed to eliminate the growth of mould.

Global composition of cheeses

Fat content (**Method 933.05, AOAC 2006**), NaCl content (**Method 975.20, AOAC 2006**) and acidity (**Method 920.124, AOAC 2006**) were measured. Dry matter and total nitrogen were determined by (**Standard IDF 4A, 1982**) and **Rossi *et al.* (2004)**, respectively. Non-protein nitrogen was determined by (**Standard IDF 25, 1964**) and fat acidity by (**Standard IDF 6A, 1969**). The pH values of the cheeses were obtained by directly inserting the tip of the probe (MV-TEMP pH meter, Digital Instruments, Taiwan) into different portions of cheese samples. Water activity (aw) was determined using a ROTRONIC a_w Quick carp instrument (New York, USA) according to manufacturer specifications.
Free fatty acids (FFA) in experimental cheeses were extracted from whole cheese fat by a column chromatographic separation step, using an alumina stationary phase and subsequent elution with solvents, according to **Deeth *et al.* (1983)**. FFA from C4:0 to C18:1 were determined by gas chromatography (GC) analysis (1:80 split injection) using a Shimadzu CG-17A gas chromatograph equipped with a flame ionisation detector (Shimadzu Corporation, Kyoto, Japan) and with a Nukol column (Supelco, Inc., Bellefonte, PA, USA, 30 m×0.25 mm ID×0.25 µm film thickness). The carrier gas was N_2 at 3 $mL.min^{-1}$ flow rate, and the oven temperature programme was 100 °C (initial temperature), 16 $°C.min^{-1}$, 210 °C (30 min). Free fatty acids were detected at day 1 (FFA_1) and at the end of ripening (FFA_{30}).

Microbiological analysis

For microbiological analysis, cheese samples (10 g) were dispersed in 90 mL of 2% (w/v) sodium citrate solution, homogenized for 2 min in a Stomacher (Laboratory Blender Stomacher model 400, Seward Medical, London, U.K.), serial dilutions in peptone saline solution were performed and plated on specific media for viable counts. Microbiological counts were performed in triplicate after 1 and 30 days of ripening. Mesophilic LAB (MRS agar pH 6.5, at 35°C for 48 h), fungi and yeasts (Yeast Extract Glucose Chloramphenicol Agar, 5 days at 30°C) and total aerobic mesophilic microorganisms (PCA, 35°C, 48 h) were determined. According to Argentinean legislation (**ANMAT, 2014**), coliforms at 30°C and 45°C (MPN method, three tubes series, Brilliant Green Lactose Bile Broth, 48h at 30°C and 45°C, respectively) were determined.

Sensorial analysis

In order to characterize the sensorial profile of cheeses at the end of ripening (30 days) they were subjected to sensorial evaluation by an internal panel consisting of 9 judges. Panellists were chosen from a group prepared and trained in sensory analysis of cheeses, having among 30–45 years old, in gender proportion of 6 male and 3 female. The members were selected to define the descriptive terminology for the sensory attributes of goat cheese in three training sessions.

Sensorial descriptive analysis was applied according to consensus technique (**IRAM, 1997; Chamorro and Losada, 2002**) and performed in three sessions with samples of each type of cheese served refrigerated (4 ± 1°C). The sensory attributes of odour and flavour were evaluated using a 5-point intensity scale ranging from less intense to more intense for most attributes. The average value of the three evaluations for each attribute of each panellist was statistically analyzed. Overall impression of the product was made considering the intensity of flavour notes present as well as the mixtures thereof. It was rated as good (high intensity), regular (medium intensity) and poor (low intensity).

Statistical analysis

Results were mean of three independent trials for each type of cheese, during a period of 3 months. Results were expressed as mean ± standard deviation (SD). ANOVA analysis (InfoStat 2011, Grupo Infostat, FCA, Universidad Nacional de Córdoba, Argentina) was carried out to determine statistical differences ($P \leq 0.05$) between samples. Tukey test was used ($P \leq 0.05$).

RESULTS AND DISCUSSION

Acidifying ability test

In cheesemaking, it has been observed that lowering the pH of milk (e.g. by the activity of starter) leads to a shorter coagulation time and a faster initial increase of gel firmness (**Brule and Lenoir, 1990**). For individual strains, the results are shown in **Table 1**. Of all studied strains, *L. plantarum* UNSE316 and *S. thermophilus* UNSE314 presented the highest acidifying activity. As *L. plantarum* strains were used as adjunct cultures they were placed in a smaller proportion in culture A. *S. thermophilus* strains, employed as starters, reached the desired pH 5.2 after 5 h of incubation, while *L. bulgaricus* strains did after 6 h of incubation.

It is generally agreed that κ-casein is predominantly located at the casein micelle surface with the hydrophobic para-κ-casein part (residues 1–105) linked to the micelle, and the hydrophilic and negatively charged caseinomacropeptide (CMP) part (residues 106–169), rich in carbohydrates, protruding into the solution. Upon hydrolysis of κ-casein by the coagulant (chymosin in this case), CMP is released, leaving para-κ-casein attached to the micelle. The removal of CMP from the micelle surface leads to a decrease in electrostatic repulsion between micelles and the steric stabilisation is also decreased. The loss of electrostatic repulsion and steric stabilisation allows attractive forces to come into play, and the micelles start to aggregate, forming a gel that eventually can expel liquid by syneresis. Aggregation is also very dependent on the concentration of calcium (**Harboe *et al*, 2010**) and the pH (**Janhøj and Qvist, 2010**). A decreasing pH leads to a decrease in the negative charge of the caseins that may favour aggregation. On the other hand, it also leads to dissolution of calcium phosphate from the casein micelles working in the opposite direction to increase the negative charge. The balance between these effects can probably explain the effect of pH on gel firming. Decreasing the pH, at least down to pH 5.3, leads to a large increase in aggregation and greatly enhances syneresis facilitating the flow of whey out of the grains (**Janhøj and Qvist, 2010**).

Table 1 Acidifying ability of the selected native lactic acid bacteria

	pH Time (hours)						
Strains	1	2	3	4	5	6	7
L plantarum							
UNSE316	6.25 ± 0.03^{D}	6.17 ± 0.02^{D}	5.97 ± 0.03^{E}	5.41 ± 0.02^{F}	5.29 ± 0.03^{E}	4.99 ± 0.02^{G}	4.97 ± 0.02^{D}
UNSE317	6.23 ± 0.03^{D}	6.19 ± 0.03^{D}	6.00 ± 0.01^{D}	5.46 ± 0.03^{E}	5.34 ± 0.02^{D}	5.04 ± 0.02^{F}	4.95 ± 0.02^{D}
L. rhamnosus							
UNSE308	6.42 ± 0.02^{AB}	6.33 ± 0.03^{B}	6.21 ± 0.03^{A}	5.85 ± 0.03^{C}	5.49 ± 0.02^{C}	5.36 ± 0.02^{C}	5.17 ± 0.03^{C}
L. bulgaricus							
UNSE309	6.44 ± 0.03^{AB}	6.29 ± 0.02^{C}	6.18 ± 0.02^{B}	5.78 ± 0.02^{D}	5.46 ± 0.02^{C}	5.28 ± 0.02^{D}	5.15 ± 0.02^{C}
P. pentosaceus							
UNSE22	6.46 ± 0.03^{A}	6.38 ± 0.03^{A}	6.21 ± 0.01^{A}	6.10 ± 0.02^{A}	5.96 ± 0.02^{A}	5.57 ± 0.02^{B}	5.40 ± 0.02^{B}
UNSE253	6.41 ± 0.02^{B}	6.36 ± 0.01^{A}	6.11 ± 0.02^{C}	6.02 ± 0.03^{B}	5.92 ± 0.03^{B}	5.65 ± 0.03^{A}	5.50 ± 0.02^{A}
S.thermophilus							
UNSE314	6.39 ± 0.01^{C}	6.27 ± 0.03^{C}	5.82 ± 0.02^{F}	5.45 ± 0.03^{E}	5.28 ± 0.01^{E}	5.05 ± 0.03^{F}	4.97 ± 0.03^{D}
UNSE321	6.41 ± 0.02^{B}	6.32 ± 0.02^{B}	6.00 ± 0.03^{D}	5.75 ± 0.02^{D}	5.34 ± 0.03^{D}	5.22 ± 0.02^{E}	5.19 ± 0.02^{C}

Legend:Values are expressed as mean ± SD.
$^{A-D}$Different letters in the same column indicate statistically significant differences ($P < 0.05$). The initial pH of milk was 6.50±0.10.

Optimal inoculation proportion test

The results obtained are shown in **Figures 1A and 1B**. Cultures S and A at 2% (v/v) reached the desired pH 5.2 in curd (see above **Acidifying ability test**) at 4 h of incubation. A four hour period from milk inoculation until para-κ-casein gel formation (optimum at pH 5.2) is considered to lead to a more efficient process of cheesemaking (**Hynes *et al*., 2000**). Therefore, the 2% (v/v) inoculum was selected for cheese manufacture in this study.

The test was also performed with the commercial culture, but C at 2% (v/v) reached the desired pH 5.2 in curd at 4.5 h of incubation (data not shown).

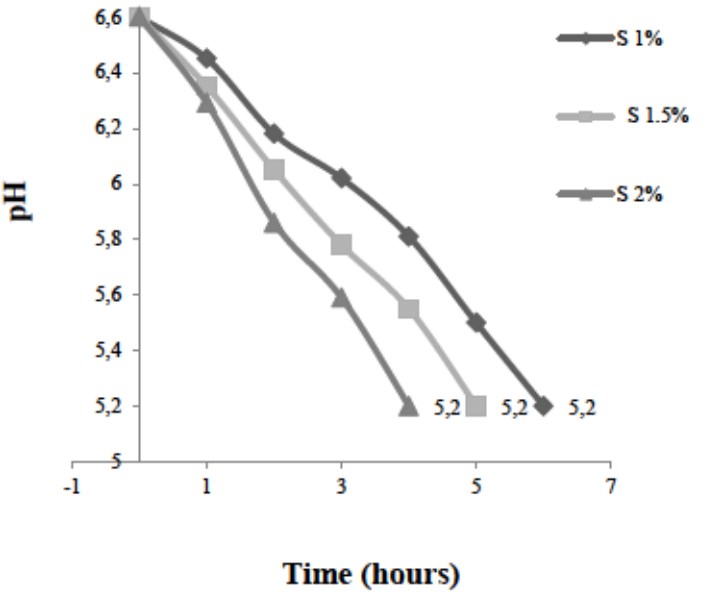

Figure 1A Acidification curve for cheesemaking with native starter culture (S) in different inoculum (1, 1.5, 2%).

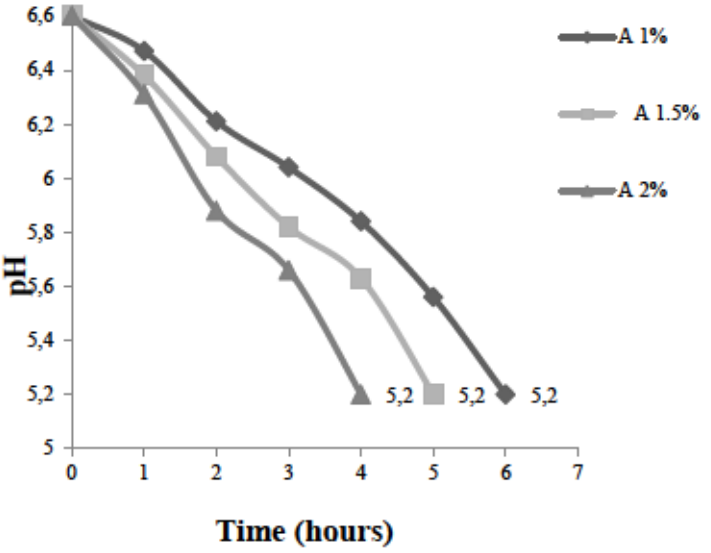

Figure 1B Acidification curve for cheesemaking with native starter plus adjunct culture (A) in different inoculum
(1, 1.5, 2%).

Chemical composition of milk

The values (means ± standard deviations) of the physicochemical parameters of the milk used in the manufacture of the experimental cheeses were % (w/v): lactose, 4.80±0.01; protein, 3.82±0.01; fat, 5.85±0.04; non-fat total solids, 9.40±0.01. Freezing point (°C), -0.523 ± 0,000, acidity, 0.17% (w/w) lactic acid; density (at 15°C) (g/ml) 1.0320 ± 0.0023 and pH was 6.5±0.10.

The composition of milk and the yield of cheese derived from a given quantity of milk is determined by a multiplicity of factors, which include the animal species (e.g. cow, goat or sheep), the breed of animal, the stage of lactation, nutrition, the lactation number and animal health (**Banks, 2007**). This study was performed in winter, when goats received rations of lower nutritional value than the rest of the year seasons and this had impact in decreased protein content. The two most important constituents with respect to cheese yield are milk fat and casein (**Banks, 2007**). According to this, the cheese yield obtained in this study was 10-11%, lower than that obtained by **Oliszewski *et al.* (2002).**

Global composition of cheese

Evolution of global composition of fresh goat cheeses during ripening is shown in **Table 2.**

Values of fat in dry matter and moisture are in accordance with the range established by Argentinean legislation (**ANMAT, 2014**) for semi fat cheeses (25.0-44.9%) and fresh cheeses (46.0-55.0%), respectively.

Results evidenced that CC, CA and CS did not show significant differences ($P< 0.05$) in: protein, fat, NaCl, acidity, fat acidity and a_w, at the same time of ripening. Fat and protein contents were close to those reported in similar type of cheese (**Franco *et al.* 2003; Janštová *et al.* 2010**).

In all experimental cheeses, titratable acidity increased progressively during ripening. Final acidity values (1.05 %) doubled those found by **López Alzogaray *et al.* (2007)** in goat milk cheeses made without selected starter addition but were slightly lower than those reported by **González and Zárate, (2012)** (1.26%) in goat cheeses made with native starter culture.

Final pH values of CS (4.92) and CA (4.88) were slightly higher than those reported by **Areti Asteri *et al.* (2010)** (4.53) in fresh cheese. Of all experimental cheeses, CC showed the highest final value of pH (5.05). pH values lower than 4.0 make the cheese very acid and maybe brittle, values higher than 5.0 are not proper and safe for good keeping quality of this type of cheese (**Alichanidis and Polychroniadou, 2008**).

In relation to fat acidity, final average values of 1.79-1.76 mg of KOH 0.1 N /g of fat (equivalent to ~3.2 mEq of acid/100 g of fat) were detected. Slightly higher value (1.92 mg of KOH 0.1 N /g of fat) was found in artisanal cheeses made with raw goat milk (**López Alzogaray *et al.* 2007**).

Table 3. shows the average concentrations of FFA determined at the end of experimental cheese ripening. The total FFA content was obtained by summing individual FFA concentrations. The values ranged from approximately 1472 to 1527 mg kg^{-1} cheese. The results are in agreement with those described for different types of goat's milk cheese. **Nouira *et al.* (2011)** found total FFA values between 1448.5 and 1495.7 mg kg^{-1} in semi-soft Cheddar-type caprine cheeses after 30 days of ripening. **Franco *et al.* (2003)** obtained a value of 1561.4 mg kg^{-1} for Babia-Laciana cheese at 15 days of ripening. **Juan *et al.* (2015)** reported similar FFA values (1444 mg.kg^{-1} cheese) in a similar type of goat's milk cheese. Total FFA contents showed significant differences between CC and cheeses made with native cultures, CS and CA.Variability in the FFA contents among replicate trials of cheesemaking was observed.

According to **Collins *et al.* (2003)**, in cheeses that underwent moderate lipolysis, FFA in the order of 2000–7000 mg kg^{-1} are liberated during ripening and make an important contribution to characteristic flavour and aroma in both raw and pasteurized milk cheese. The lipolysis degree was determined as ratio total FFA_{30}/FFA_1. The highest ratio was detected in CA.The FFA profile of experimental cheeses indicated that the most abundant acids were palmitic (C16:0), oleic (C18:1), stearic (C18:0), capric (C10:0) and lauric (C12:0) (in decreasing order), representing together about 77.6% of the total FFA content. A similar pattern has been reported by **Juan *et al.* (2015)**.

Proteolysis is the most complex event that occurs during ripening and gives a significant contribution to flavour, via the formation of amino acids and small peptides (**Fox and Cogan, 2004**). Experimental cheeses underwent a slight proteolysis since NPN values were lower (4.54- 4.49%) than those reported in cheeses subject to moderate proteolysis (18.20%) (**Fontecha *et al.* 1990**) but higher than those found by **Olarte *el al.* (2000)** (4.12%) and **Albenzio *et al.* (2006)** (0.2-0.4%). Of all experimental cheeses, CC showed the lowest levels of proteolysis during ripening.

The salt level markedly influences cheese flavour and aroma and, hence, overall quality (**Guinee, 2004**). Average values of salt content (1.27%) were in accordande with those reported in most of cheeses (0.5 to 2%) (**Hardy, 1990**). As a consequence, the cheesemaking protocol described in this work allowed the successful control of the factors that affect salt uptake and distribution in cheese. Among these factors, the most important are the pH and aw of the cheese, temperature and concentration of the brine and standing time in brine (**Hardy, 1990**). Thus moderate values of salt content were measured without affecting the cheese overall quality.

Table 2 Global composition of experimental fresh goat cheeses at the beginning and end of ripening.

Days of ripening	Cheeses[a]	Global composition of experimental cheeses									
		Protein (% TS)	Fat (% w/w)	Total solids (% w/w)	NaCl (% w/w)	pH	Titratable acidity g of lactic acid/100g of cheese	NPN (%TN)	Fat acidity mg KOH 0,1N/ g of fat	Moisture (%, w/w)	aw
1	CS	32.73±0.30^{B}	30.27±0.32^{B}	48.52±0.30^{B}	0.98 ±0.02^{B}	5.22±0.20^{A}	0.75±0.07^{B}	1.48±0.34^{C}	0.180±0.007^{A}	51.80±0.62^{A}	0.992±0.004^{B}
	CA	32.80±0.32^{B}	30.52 ±0.28^{B}	48.73±0.33^{B}	0.96±0.02^{B}	5.20±0.28^{A}	0.73±0.04^{B}	1.43±0.22^{C}	0.182±0.005^{A}	51.83±0.56^{A}	0.991±0.003^{B}
	CC	32.48±0.20^{B}	30.12 ±0.36^{B}	48.40±0.23^{B}	0.97 ±0.01^{B}	5.24±0.26^{A}	0.70±0.07^{B}	1.41±0.20^{C}	0.183±0.005^{A}	51.78±0.56^{A}	0.990±0.002^{B}
30	CS	34.90±0.30^{A}	33.70 ±0.39^{A}	52.50±0.30^{A}	1.29 ±0.02^{A}	4.92±0.30^{C}	1.05±0.07^{A}	4.54±0.22^{A}	1.790±0·006^{B}	49.71±0.53^{B}	0.979±0.004^{A}
	CA	34.98±0.25^{A}	33.83 ±0.35^{A}	52.55±0.28^{A}	1.27 ±0.03^{A}	4.88±0.32^{C}	1.04±0.07^{A}	4.49±0.22^{A}	1.782±0·005^{B}	49.76±0.42^{B}	0.980±0.003^{A}
	CC	34.68±0.22^{A}	33.23±0.33^{A}	52.68±0.28^{A}	1.25 ±0.02^{A}	5.05±0.24^{B}	1.02±0.06^{A}	4.18±0.22^{B}	1.757±0·008^{B}	49.68±0.35^{B}	0.978±0.004^{A}

Legend:Values are mean of three trials for each type of cheese. Different letters in each column at the same time of ripening indicate statistically significant differences (P<0.05) between experimental cheeses.
[a]Experimental cheeses, CC (cheese manufactured with commercial culture C), CS (cheese manufactured with autochthonous starter culture S), CA (cheese manufactured with autochthonous starter and adjunct culture A).
Titratable acidity values are expressed as g of lactic acid/100 g of cheese. Fat, Total solids, NaCl and Moisture values are expressed as g/100 g of cheese. Protein values are expressed as percentage of Total solids. Non protein nitrogen (NPN) values are expressed as percentage of total nitrogen.

Table 3 Free fatty acid concentrations of experimental fresh goat cheeses at end of ripening

	Type of cheese[a]		
FFA group[b]	CS	CA	CC
SCFA	138 ± 20^{A}	144 ±18^{A}	126±31^{B}
MCFA	407±50^{A}	411±42^{A}	390±38^{B}
LCFA	963 ±60^{A}	972 ±54^{A}	956±45^{A}
Total FFA	1.508±21^{A}	1.527±28^{A}	1.472±27^{B}
Ratio total FFA_{30}/FFA_{1}	2.90	2.94	2.88

Legend: Values are mean of three trials for each type of cheese. Different letters in each row indicate statistically significant differences (P<0.05) between experimental cheeses.
FFA, Free fatty acids concentrations are expressed in $mg.kg^{-1}$ cheese.
FFA_{30}, free fatty acid concentration at the end of ripening; FFA_{1}, free fatty acid concentration at day 1.
[a] CS, cheese manufactured with native culture S; CA, cheese manufacture with native culture A; CC, cheese manufactured with commercial culture C.
[b] SCFA, short chain fatty acids (C4:0–C8:0); MCFA, medium-chain fatty acids (C10:0–C14:0), LCFA, long-chain fatty acids (C16:0–C18:2).

Microbiological analysis

Evolution of microorganisms in experimental fresh goat cheeses during ripening is shown in **Table 4**.
Results evidenced that CC, CA and CS did not show significant differences (P < 0.05) in total counts of aerobic mesophilic microorganisms and coliforms at 30°C, at the same time of ripening. Total coliforms per gram at 30°C showed counts below the maximum allowed by Argentinean legislation (m=5000; M=10000), for cheeses of high humidity (46.0-55.0% moisture) (**ANMAT, 2014**), coliforms at 45°C were not detected; these results confirm that the manufacturing protocol used allowed making microbiologically safe cheeses. At the end of ripening, there were no significant differences in mesophilic LAB counts and fungi and yeast counts between experimental cheeses. Microbial counts showed a growth of LAB in all experimental cheeses between 8.02 and 8.34 log CFU g^{-1}. These results indicate that LAB remained viable during ripening thus contributing to the moderate lipolysis and slight proteolysis that were detected in all cheese samples.

Table 4 Counts of the main microbial groups during ripening of experimental fresh goat cheeses.

Day of ripening	Cheeses*	Microbial counts in experimental cheeses: Lactic acid bacteria (log CFUg^{-1})	Total mesophilic (log CFU g^{-1})	Coliforms at 30°C (MPN/g)	Fungi and yeasts (log CFU g^{-1})
1	CS	7.54±0.35^{B}	4.04±0.25^{B}	3.09±0.14^{A}	5.27±0.20^{B}
	CA	7.86±0.38^{B}	4.10±0.30^{B}	3.13±0.26^{A}	5.38±0.30^{B}
	CC	6.98±0.33^{C}	4.12±0.20^{B}	3.17±0.22^{A}	5.30±0.22^{B}
30	CS	8.26±0.25^{A}	4.18±0.30^{A}	2.18±0.16^{B}	5.40±0.20^{B}
	CA	8.34±0.42^{A}	4.26±0.23^{A}	2.22±0.12^{B}	5.48±0.30^{B}
	CC	8.02±0.33^{A}	4.30±0.25^{A}	2.29 ±0.18^{B}	5.67±0.32^{B}

Legend: Values are mean of three trials for each type of cheese. Counts of microbial groups are expressed as log CFUg^{-1}. Different letters in each column at the same time of ripening indicate statistically significant differences ($P<0.05$) between experimental cheeses. *Experimental cheeses, CS (cheese manufactured with autochthonous starter culture), CA (cheese manufactured with autochthonous starter and adjunct cultures) and CC (cheese manufactured with commercial culture).

Sensorial analysis

The sensory profiles of the three types of cheeses are shown in **Figures 2A** and **2B**. The results of the sensory evaluation showed that CA received the highest score, followed by CS, in comparison with CC at 30 days of ripening. The CA and CS showed significant differences for odour attributes as fresh milk, fruity and propionic acid. CA presented the highest score for fruity and propionic acid attributes, as well as stronger goaty note than commercial cheese (**Fig. 2A**). Respect to flavour (**Fig. 2B**), all types of cheeses showed significant differences for flavour attributes as cream, spiciness propionic acid and bitterness. CA was highlighted by its highest score for cream and spiciness and lowest score for bitterness attributes. Overall impression of cheeses elaborated with native cultures (CS and CA) was qualified as good compared to CC that was scored as regular.

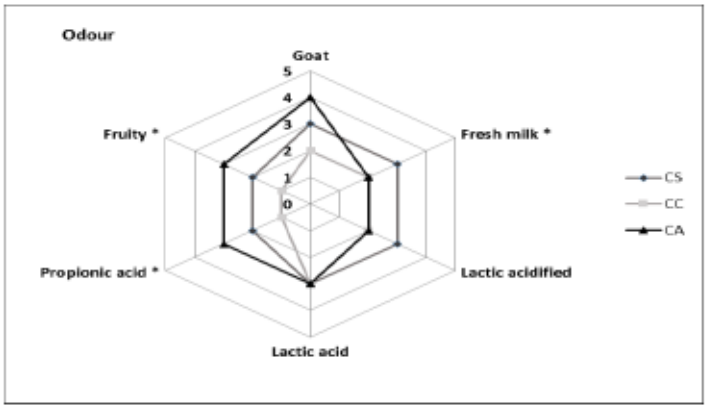

Figure 2A Quantitative sensory profile of three types of fresh goat cheeses (CS, CA,CC) at the end of ripening (30 days)(*$p < 0.05$): (A) odour attribute.

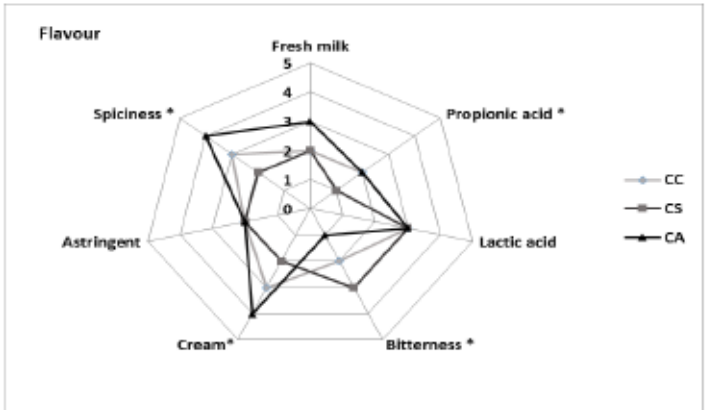

Figure 2B Quantitative sensory profile of three types of fresh goat cheeses (CS, CA, CC) at the end of ripening (30 days)(*$p < 0.05$): (B) flavour attribute.

CONCLUSION

Fresh goat cheeses made with both native and commercial cultures presented moderate lipolysis and slight proteolysis during ripening (30 day-old-cheese), obtaining a product of similar global composition. Native cultures S and A showed a better perfomance in cheesemaking.

The fresh goat cheeses made with native cultures S and A were greater scored for their suitable typical flavour and satisfying overall sensorial characteristics. These results should be of great interest for the eventual manufacture of this type of cheese on an industrial scale. However, more studies are needed to conveniently perform the scaling up.

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ANTIOXIDANT ACTIVITY OF PHENOLS AND FLAVONOIDS CONTENTS OF AQUEOUS EXTRACT OF *PELARGONIUM GRAVEOLENS* ORGIN IN THE NORTH-EAST MOROCCO

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ABSTRACT

The objective of this work is to characterize the phenols and flavonoids compounds of extracts of ***Pelargonium Graveolens*** from North-East Morocco (TAZA) in terms of antioxidant activity. The antioxidant activity of this aromatic plant was determined according to the DPPH radical scavenging assay to suggest it as a new potential source of natural antioxidants. The quantification of phenolics and flavonoids compounds of solvent extracts (diethyl ether and ethyl acetate) were determined spectrometrically. The DPPH scavenging activity of extracts increased in the order diethyl ether extract < ethyl acetate extract < ascorbic acid. Based on these results, we suggest that the phenols and flavonoids compounds of ***Pelargonium Graveolens*** have significant potential as a natural antioxidant.

Keywords: *Pelargonium Graveolens*, Phenols, Flavonoids, DPPH method, Antioxidant activity

INTRODUCTION

The oxidative degradation of food by free radicals, causes a real problem to the health of the consumer, so it is necessary to use food additives, called "antioxidants" to fight against this phenomenon. Many substances from aromatic and medicinal plants have been shown to contain antioxidants like flavonoid compounds, these Compounds are the main agents that can donate hydrogen to free radicals and thus break the chain reaction of lipid oxidation at the first initiation step (**Agraval, 1989**). This high potential of phenolic constituents to scavenge radicals may be explained by their phenolic hydroxyl groups (**Havsteen, 2002**). Several studies exhibited a strong relationship between total phenolic content and antioxidant activity in fruits, vegetables, and medicinal plants (**Ouariachi *et al.*, 2004**).
The antioxidant activity of a compound is its ability to resist oxidation. The best known antioxidants are the β-carotene (provitamin A), ascorbic acid (vitamin C), tocopherol (vitamin E) as well as phenolic compounds. Indeed, the most synthetic antioxidants or naturally occurring groups have phenolic hydroxyl in structure and antioxidant properties are attributed in part, to the ability of these natural compounds to scavenge free radicals such as hydroxyl radicals (OH•) and superoxide (O_2•) (**Rice-Evans *et al.*, 1995; Ouadi *et al.*, 2015; Ghazi *et al.*, 2015; Hazi *et al.*, 2015; Saidi *et al.*, 2016**). Pelargonium Graveolens is a shrub of the family Geraniaceae, this Moroccan medicinal plant, known locally as "LAATARCHA", is used for the treatment of various inflammatory disorders such as: Antispasmodic, Relaxing and Anti-inflammatory. The aim of this study is to investigate the antioxidative properties of solvent extracts of Pelargonium Graveolens from North East of Morocco (TAZA). Additionally, the total phenolic and flavonoid contents of diethyl ether and ethyl acetate extracts have been determined.

MATERIAL AND METHODS

Plant material

The aerial part of *Pelargonium Graveolens* was harvested in April 2013 in TAZA at the North-East of Morocco. A voucher specimen was deposited in the Herbarium of Faculty of Sciences, Oujda, Morocco. The dried plant material is stored in the laboratory at room temperature (298 K) and in the shade before the extraction. The different proprieties of *Pelargonium Graveolens* are:
Scientific name: Pelargonium Graveolens; Common name: Géranium-Rose; Local Name: Laâtarcha; Order: Geraniales; Family : Geraniaceae; Genre : Pelargonium; Kingdom : plantae; Division : Magnoliophyta; Class :Magnoliopsida and Flowering: August-January with a peak in September-October (**Demarne, 2002; Van der Walt and Andri. 1992**) (figure 1).

Figure 1 Pelargonium; Kingdom

Fractionating the aqueous extract

Polyphenols present in the aqueous extract, are extracted after filtration by two solvents of different polarity namely, ethyl acetate and diethyl ether, polarity indices respectively, 0.58 and 0.38. A sample of 100 ml of the extract recovered by steam distillation is hydrolyzed with 40 ml 2N HCl bath at 100 °C for one hour, at the end of this treatment, plant debris clusters are formed and extract aqueous recovered is filtered and then mixed in a separating funnel and shaken thoroughly with diethyl ether or ethyl acetate. After settling in an ampoule, the upper organic phase is collected in an Erlenmeyer flask, the extraction is repeated 3 times with solvent renewal. The latter is due evaporated after drying the organic phase with anhydrous sodium sulfate, and the resulting extract is considered as the fraction of diethyl ether or ethyl acetate. The fractions thus obtained were stored in glass vials and then kept at a temperature of 4 to 5 °C prior to analysis.

Determination of total phenolics contents

The ployphénols are estimated by various methods such as the method of Prussian blue (**Graham,1992**), but the most used is the Folin-Ciocalteu. This consists of a mixture of phosphotungstic acid (H3PW12O40) and phosphomolybdic acid (H3PMo12O40); it is reduced by the phenols in a mixture of the blue oxides of tungsten and molybdenum (**Boizot and Charpentier, 2006**). Moreover, 1ml of Folin reagent (diluted 10 times in distilled water) was added to 200 µl of sample or standard (gallic acid) with suitable dilutions in distilled water, after 4 min, 800 µl of a solution sodium carbonate (75 mg/ml) are added to the reaction medium. After 45 minutes incubation at room temperature, the absorbance of the resulting solution is measured at 760 nm. The same procedure was also applied to the standard solutions of gallic acid, and a standard curve was obtained. The concentrations of phenolic compounds expressed as µg gallic acid equivalent per mg of extract were calculated according to the standard gallic acid graph. All experiments were performed in triplicate assays, and gallic acid equivalent values were reported as X (average) ± SD (standard deviation) of triplicates (**El Ouadi *et al.*, 2015**).

Determination of total flavonoids contents

Quantification of flavonoids in extracts of *Pelargonium Graveolens* were performed by the method of aluminum trichloride (**Bahorun *et al.*, 1996; Arvouet-Grand *et al.*, 1994**). In addition, 1 ml of sample or standard (dissolved in methanol) was added to 1 ml of the solution of $AlCl_3$ (2% in methanol). After 30 minutes of reaction, the absorbance is read at 415 nm. The concentrations of flavonoid compounds expressed as µg rutin equivalent per mg of extract were calculated according to the standard rutin graph. All experiments were performed in triplicate assays and rutin equivalent values were reported as X ± SD of triplicates.

Antioxidant activity

The free radical-scavenging activities of solvent extracts were measured using 1,1-diphenyl-2-picrylhydrazyl(DPPH) as described by researchers (**Hatano *et al.*, 1985**); antioxidants react with the stable free radical DPPH (deep violet color) and convert it to 1,1-diphenyl-2-picrylhydrazine with discoloration.

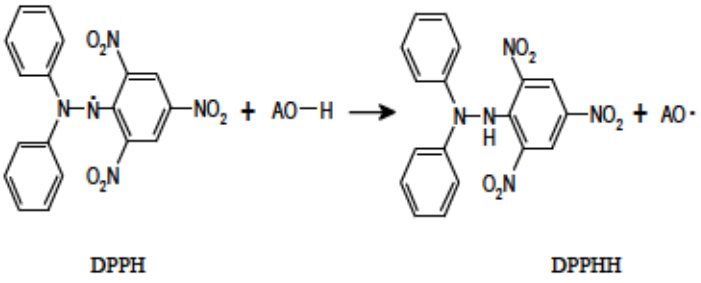

Figure 2 antioxidant activity of DDPH

Where: (AO-H) represents a compound capable of yielding hydrogen to DPPH radical (violet) to transform it into picryl diphenyl hydrazine (yellow) [16]. Various different concentrations prepared in ethanol for the different samples and standard studied are between 0.2 to 2 µg/ml were added to 3.9 ml of a DPPH radical solution in ethanol. The mixture was strongly shaken and left to stand at room temperature for 30 min in the dark. The absorbance was measured at 517 nm against a blank. The radical-scavenging activity was expressed as percentage of inhibition (I%) according to the following formula [17]:

$$I\,(\%) = 100^{*}\,(A_{control} - A_{sample})/A_{control}$$

Where $A_{control}$ is the absorbance of the control reaction and A_{sample} is the absorbance of the test compound. Thesample concentration providing 50% inhibition (IC50) was calculated from the graph of inhibition percentage against sample concentration. Tests were carried out in triplicate. Ascorbic acid was used as a positive control.

RESULTS AND DISCUSSION

Total phenolic and flavonoid contents of solvent extracts

The determination of levels of total phenols and flavonoids in two fractions of aqueous extract of *Pelargonium Graveolens* was made by using separately methods colorimetric (Folin-Ciocalteux and trichloride aluminum (AlCl3)).
The content of total phenols estimated by the Folin-Ciocalteu method for each extract fraction was reported in µg gallic acid equivalent / mg of extract. The results show that the fraction of ethyl acetate has a high content of total phenols (437± 25 µg/mg) compared to that of the fraction diethyl ether (400 ± 29 µg/mg) (Table 1).
The flavonoid content determined by the method trichloride alumiunm of each extract fraction was reported in µg equivalent rutin / mg of extract. The results reveal that ethyl acetate and diethyl ether fractions of aqueous extract of *Pelargonium Graveolens* have respectively moderate levels (Table 1) (29±1,6 and 12±0,2 µg equivalent of rutin per mg of extract). Figures 3 and 4, show the calibration curves of gallic acid and rutin.

Table 1 Determination of total polyphenols and flavonoids in both fractions extracts of *Pelargonium Graveolens*

	Extract	polyphenols in µg equivalent of gallic acid per mg of extract	flavonoids in µg equivalent of rutin per mg of extract
Pelargonium Graveolens	Fraction diethyl ether	400 ± 29	12± 0.2
	Fraction ethyl acetate	437± 25	29± 1.6

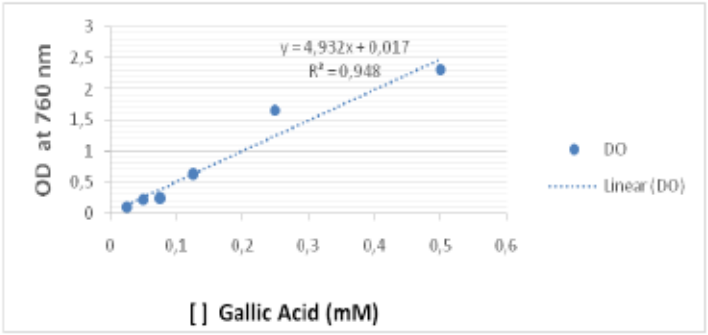

Figure 3 Calibration curve of Gallic Acid

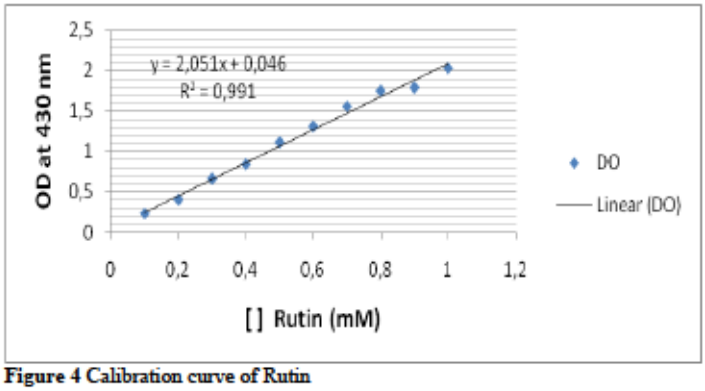

Figure 4 Calibration curve of Rutin

Antioxidant Activity

Results of free radical scavenging activity of fraction diethyl ether, fraction ethyl acetate and Acid Ascorbic (positive control) are given in Table 2. Moreover, the examination of Table 2, we spotted that the DPPH scavenging activities (%) were increased significantly with increasing the concentration of the studied samples from 0.2 to 2 µg/ml.

Table 2 The antioxidant activity of the fractions (diethyl ether and ethyl acetate) of the aqueous extract of ***Pelargonium Graveolens*** at different concentrations

Echantillons	Antioxidant activity						
Fraction diethylether	Concentrations of the extract (µg/ml)	0.2	0.35	0.5	1	2	
	Effet of trapping on DPPH (%)	12.07	21	28.31	43	51.84	
	DPPH IC_{50} (µg/ml)						1.54
Fraction acetate d'ethyle	Concentrations of the extract (µg/ml)	0.2	0.35	0.5	1	2	
	Effet of trapping on DPPH (%)	13.24	22.70	30.01	45	53	
	DPPH IC_{50} (µg/ml)						1.49
ascorbic Acid	Concentrations of the extract (µg/ml)	0.2	0.35	0.5	1	2	
	Effet of trapping on DPPH (%)	20	28	32	55	83	
	DPPH IC_{50} (µg/ml)						0.90

From Table 2, the antioxidant activity of ethyl acetate fraction is greater than the diethyl ether fraction. This activity of the extracts increases with the concentration, this is explained by the fact that the studied samples give hydrogen to DPPH who then converted to the color violet in yellow and absorbs less light. When the concentration is high, more antioxidants DPPH is reduced, so less it absorbs light passing through it (**El Ouadi *et al*., 2015**)

Generally, *Pelargonium Graveolens* shows some good antioxidant activity at the concentration of 2 µg/ml reach until 53% and 51.84% for the ethyl acetate fraction and diethyl ether fraction respectively (Figure 5). The two fractions of *Pelargonium Graveolens* extracts exhibit a lower activity than that of ascorbic acid (83%). The ethyl acetate fraction had the highest radical scavenging activity with the lowest IC50 value (1.49 µg/ml). This value was higher with diethyl ether fraction (IC50 is 1.54 µg/ml). Additionally, the fraction of ethyl acetate has a lower scavenging capacity than ascorbic acid (IC50 is 0.90 µg/ml).

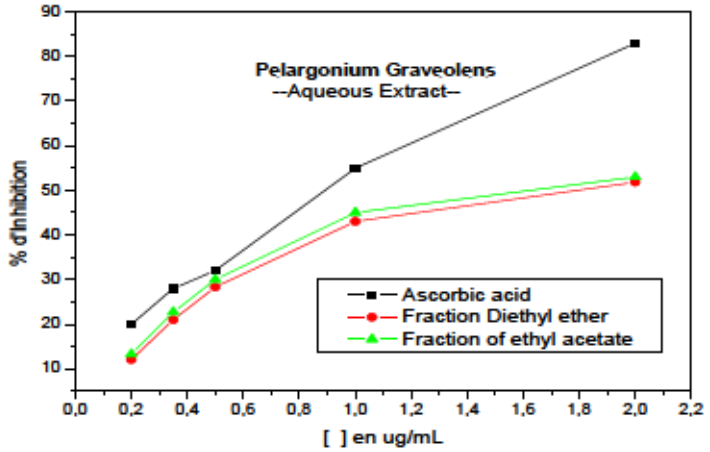

Figure 3 Antioxidant power of two fractions of the aqueous extract of *Pelargonium Graveolens*, OD reading after 30 min of incubation.

CONCLUSION

The test DPPH• is considered a simple, quick and easy to implement, experiments have shown some difficulties in measuring the reduction of state: a dynamic phenomenon at low concentrations and accompanied many compounds formed in some cases unstable. The DPPH• test is not quantitative, it compares different extracts then according to their ability to scavenge DPPH• and thus to appreciate the qualitative changes in phenolic compounds. Evaluation of the antiradical activity should be interpreted with caution, knowing that the absorbance of DPPH• at 515-520 nm decreases under the action of light, oxygen, depending on the pH and the type of the solvent added to the antioxidant. According to the results obtained, there is a relationship between the total polyphenol content and antioxidant activity. The both fractions of the *Pelargonium Graveolens* aqueous extract can reduce the radical 2,2-diphenyl-1-picrylhydrazyl, so they have an antioxidant effect in vitro, and can be proposed as new potential sources of natural additives in the food and pharmaceutical industries.

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